AD_____

Award Number: W81XWH-09-1-0650

TITLE: Cellular consequences of telomere shortening in histologically normal breast tissues

PRINCIPAL INVESTIGATOR: Christopher M. Heaphy, Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21218-2680

REPORT DATE: September 2013

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
September 2013	Annual Summary	1 September 2009 – 31 August 2013

4. TITLE AND SUBTITLE

Cellular consequences of telomere shortening in histologically normal breast tissues

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0650

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Christopher M. Heaphy, Ph.D.
Pedram Argani, M.D.
Alan K. Meeker, Ph.D.
E-Mail: cheaphy@jhmi.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
Baltimore, MD 21218-2680

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Three independent sets of normal breast tissues without evidence of cancer were analyzed. These sets were either obtained from patients undergoing reduction mammoplasty or from women at time of autopsy. The postdoctoral trainee has shown that moderate to dramatic telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in the majority of histologically normal terminal ductal lobular units. However, the extent and degree of telomere shortening varies by the individual. These data imply that there is a reservoir of genetically altered, yet histologically normal, cells within normal breast tissues that represents fertile ground for tumor development. In other telomere biology related studies, the trainee has (i) determined that the ALT phenotype (a telomere maintenance mechanism) is prevalent in 2% of breast carcinomas, (ii) demonstrated that alterations in two genes, ATRX and DAXX, which participate in chromatin remodeling at telomeres are closely associated with the ALT phenotype, and (iii) showed that telomere lengths among cancer cells and cancer-associated stromal cells is strongly associated with progression to metastasis and cancer death. In addition, the training environment has provided grounding in both basic and translational breast cancer research for the trainee. The interactive, multidisciplinary research environment at Johns Hopkins has provided the investigator opportunities to interact with pathologists, oncologists and epidemiologists, thus fostering his future success as an independent translational breast cancer researcher.

15. SUBJECT TERMS
ALT, Breast cancer, Cellular senescence, DNA damage, Telomere, Terminal ductal lobular unit

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	59	

Table of Contents

INTRODUCTION

My overall research goals are to further our understanding of the contribution of telomere biology in cancer development and progression and to use this knowledge to identify new biomarkers for the accurate prediction of cancer risk, early detection, and prognosis. Ideally, these biomarkers would have utility both at the population level and for an individual patient. As a basic scientist, I have been working to elucidate the mechanisms of tumor initiation and progression (e.g telomere length alterations), as well as understanding how the interactions between the tumor and its' tissue microenvironment may contribute to this process. Independent investigations, including from our own laboratory, have demonstrated the existence of cells with shortened telomeres in histologically normal tissues (*Meeker et al, 2004; Kurabayashi et al, 2008*). In this proposal, we determined that telomere shortened normal cells occur in all breast specimens we assessed, even in the absence of a nearby tumor. In addition, we characterized the spectrum of cellular consequences of these telomere shortened normal cells. Furthermore, other telomere biology related studies were pursued involving exciting new data involving the Alternative Lengthening of Telomeres (ALT) pathway, a telomerase-independent telomere maintenance mechanism. In addition to the scientific investigations, this award has provided the trainee opportunities to interact with pathologists, oncologists, and epidemiologists to learn (i) normal and abnormal breast morphology, (ii) the strengths and limitations of currently used breast cancer biomarkers, (iii) current standards of breast cancer treatment, and (iv) the scientific rationale for ongoing clinical trials. These interactions have helped foster his future success as an independent translational breast cancer researcher.

BODY

Summary of timeline: This BCRP Postdoctoral Training Award was initiated with a September 1, 2009 start date. Since the proposal included the use of human subjects, we wrote and received approval from the Office of Human Subjects Research Institutional Review Board at Johns Hopkins (November 12, 2009) and from the Human Research Protection Office of the U.S. Army Medical Research and Materiel Command (January 27, 2010) for collection of the clinical samples to be used in this investigation. A one year no-cost extension was requested (June 19, 2012) and granted (June 29, 2012). This Training Award recently ended (August 31, 2013).

Tissue Collection: During Year 1, collection protocols for clinical specimens were established for fluorescent *in situ* hybridization (FISH), immunofluorescence (IF) and immunohistochemistry (IHC) experiments that utilize formalin-fixed, paraffin-embedded (FFPE) tissues. Protocols were also established for primary cell culture experiments that utilize freshly collected human breast tissue. In total, histologically normal breast tissue from 1cm and 5cm away from the visible tumor margin was obtained from 57 women undergoing radical mastectomy. Additionally, histologically normal breast tissue from the right and left breast was obtained from 23 women undergoing bilateral reduction mammoplasty. FFPE tissue blocks were generated for all of these clinical specimens. In addition, using published protocols [1], primary cell cultures were established from 24 of the women undergoing radical mastectomy and from 8 women undergoing reduction mammoplasty.

Results: Using the FFPE specimens obtained from the 23 reduction mammoplasty specimens outlined above, telomere lengths were determined using the telomere-specific FISH assay developed in our laboratory. As shown in Figure 1, telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in histologically normal terminal ductal lobular units (TDLU). In some TDLUs, the luminal cells, negative for smooth muscle actin (SMA), show comparable telomere intensities similar to the adjacent myoepithelial cells (**panel A**). In contrast, some TDLUs demonstrate dim telomere signals in the luminal cells when compared to the adjacent myoepithelial cells (**panel B**). Through digital image analysis, quantitative determination of the telomere FISH signals confirms this moderated telomere shortening (**panel C**). Strikingly, telomere shortening occurs in the majority of histologically normal TDLUs analyzed from patients undergoing reduction mammoplasty, but the extent and degree of shortening varies by the individual.

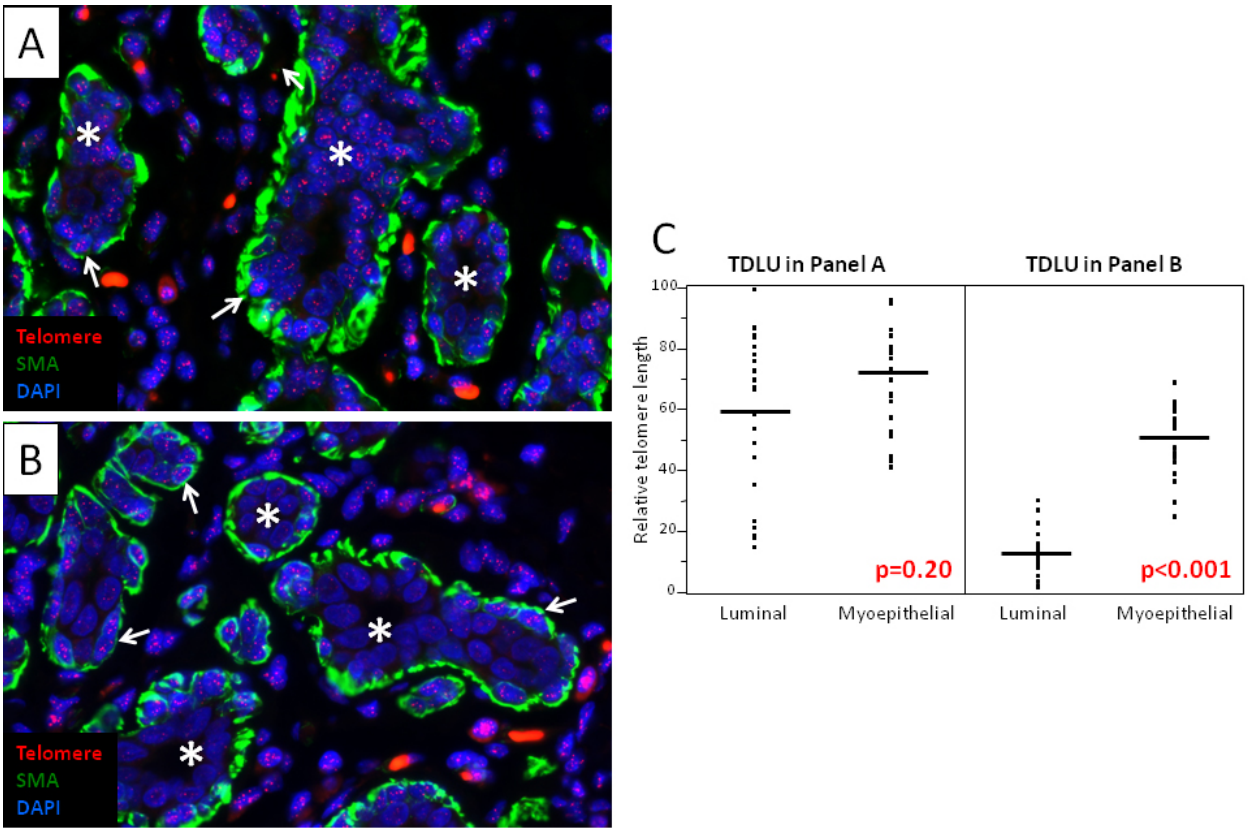


Figure 1. Telomere-specific FISH in normal breast tissues obtained from women undergoing reduction mammoplasty surgeries*. (A) A normal breast TDLU with normal length telomeres in all cell types present. (B) A normal breast TDLU with short telomeres in the luminal cells. The asterisks (*) show luminal cells and the white arrows show myoepithelial cells demarcated by the presence of smooth muscle actin (green). Telomeres (red) and DAPI-stained nuclei (blue) are also shown. (C) Quantification by digital image analysis of relative telomere lengths by determining the mean DAPI-normalized telomere signal intensities in 25 randomly selected luminal and myoepithelial cells.*

Since telomere shortening has been linked to age and all the women in the reduction mammoplasty cohort were relatively young, we sought to assess another cohort of normal breast tissues obtained from women. To accomplish this, we collaborated with Dr. Mark Sherman (Division of Cancer Epidemiology & Genetics; National Cancer Institute) to obtain normal breast tissues from 7 women at the time of autopsy. As observed in the previous cohort, telomere shortening occurred in the majority of histologically normal TDLUs analyzed from these women; again, the extent and degree of shortening varied by the individual (**Figure 2**).

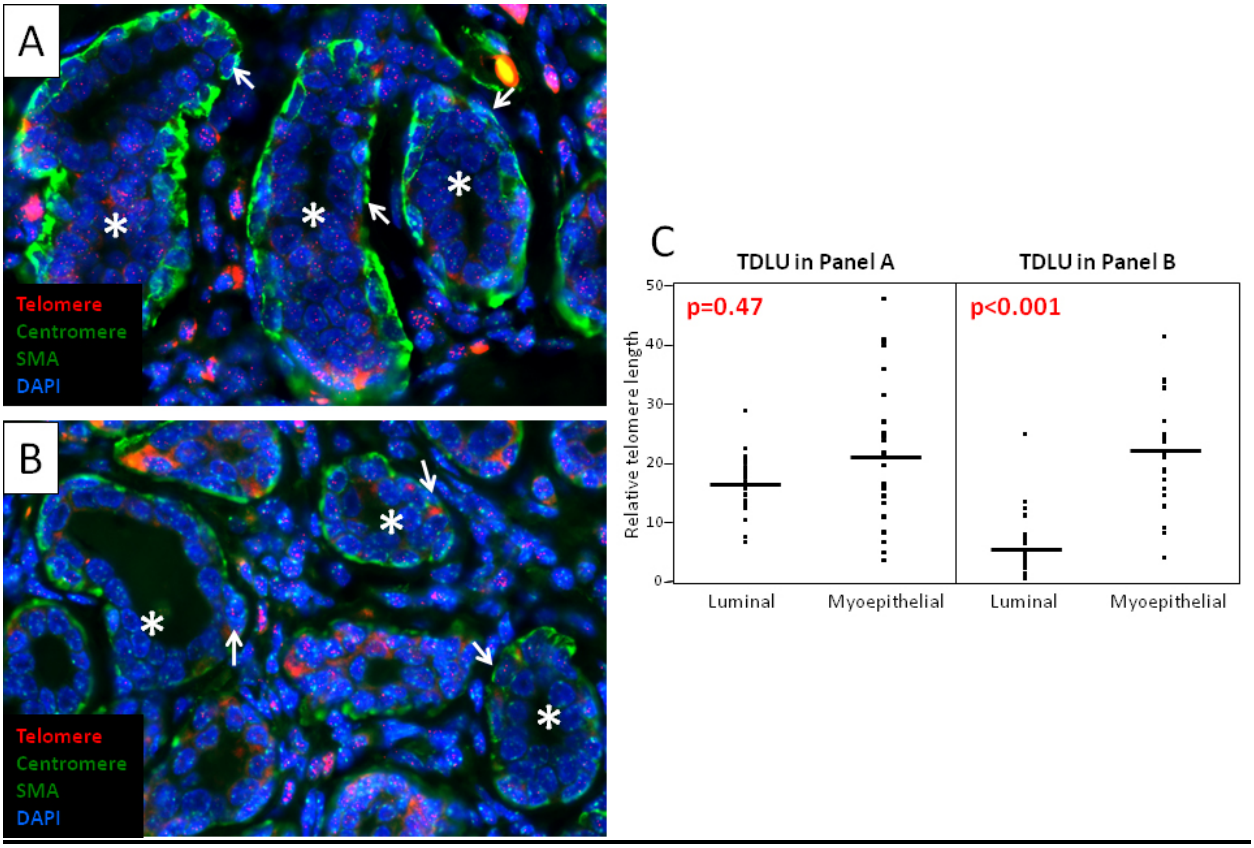


Figure 2. Telomere-specific FISH in normal breast tissues obtained from a woman at time of autopsy. (**A**) *A normal breast TDLU with normal length telomeres in all cell types present.* (**B**) *An adjacent normal breast TDLU with short telomeres in the luminal cells. The asterisks (*) show luminal cells and the white arrows show myoepithelial cells demarcated by the presence of smooth muscle actin (green). Telomeres (red), centromeres (green) and DAPI-stained nuclei (blue) are also shown.* (**C**) *Quantification by digital image analysis of relative telomere lengths by determining the mean DAPI-normalized telomere signal intensities in 25 randomly selected luminal and myoepithelial cells.*

Finally, we sought to validate our findings in a third, independent cohort of normal breast tissues obtained from women without evidence of cancer. To accomplish this, we collaborated with Dr. Kala Visvanathan (Department of Epidemiology; The Johns Hopkins Bloomberg School of Public Health) to obtain normal breast tissues from eleven women obtained by reduction mastectomy. As observed in the previous two sets, telomere shortening occurred in the majority of histologically normal TDLUs analyzed from these women; again, the extent and degree of

shortening varied by the individual. Results from the three independent sets are summarized in Table 1.

Table 1. Summary of the three independent sets of normal breast tissue with regards to the presence of telomere shortening in the luminal epithelial cell compartment.

Set	Tissue Type	N	# of cases with luminal telomere shortening (% of all cases)
Total Set		41	41 (100%)
Hopkins	Reduction Mammoplasty	23	23 (100%)
NCI	Autopsy	7	7 (100%)
Bloomberg SoPH	Reduction Mammoplasty	11	11 (100%)

In summary, moderate to severe telomere shortening is highly prevalent within histologically normal TDLUs obtained from women undergoing reduction mammoplasty surgeries and in women at time of autopsy. The dramatic telomere shortening specifically occurs in luminal epithelial cells, but not in myoepithelial cells. All women examined in the 3 independent sets contained some luminal telomere shortening in their normal TDLUs, but the extent and degree of luminal telomere shortening varied by the individual. These data will be included in a manuscript (*in preparation*).

Since the overall goal of our research is to determine the role telomere biology plays in the initiation and progression of human breast cancer, in addition to the studies in normal, cancer-free breast tissues, we also assessed telomere lengths in breast tumors. Telomere lengths were evaluated in invasive breast cancer cases (N=103) and the presence of short cancer cell telomere lengths were associated with the more aggressive breast cancer subtypes, (eg. HER-2 positive and triple-negative tumors), suggesting tumor telomere length may have clinical utility as a prognostic and/or risk biomarker [2].

Dysfunctional telomeres cause genomic instability via chromosomal breakage-fusion-bridge cycles. In the majority of human cancers, telomere dysfunction is attenuated through up-regulation of the enzyme telomerase. However, telomere loss may also be compensated in some cancers by the telomerase-independent telomere maintenance mechanism termed Alternative Lengthening of Telomeres (ALT).The ALT phenotype has rarely been reported in epithelial malignancies; however, our laboratory previously reported the presence of ALT in a small subset of invasive breast carcinomas [3]. We confirmed this finding by assessing a total of 377 breast carcinomas and observed the ALT phenotype in 7 cases (2%). In addition to the breast data, we comprehensively surveyed the ALT phenotype in 6,110 primary tumors from 94 different human cancer subtypes. Overall, the prevalence of the ALT phenotype was 3.73%; however, the prevalence varied vastly between different subtypes. ALT-positive cancers are predicted to be resistant to anti-telomerase therapies; these findings may have therapeutic implications [4].

Since the ALT pathway plays a critical role in tumorigenesis in certain tumor types, it was interesting to our group that two genes, *ATRX* and *DAXX*, that participate in chromatin remodeling at telomeres were found to be mutated at a high rate in pancreatic neuroendocrine tumors (PanNETs); a tumor type that contains a high proportion of tumors displaying the ALT

phenotype [5]. In collaboration with this group, breast tumor genomic DNA (N=96) was sequenced for *ATRX* and *DAXX*. Unfortunately, we did not observe any mutations in these two genes. However, we did observe that all of the PanNETs that exhibited the ALT phenotype had *ATRX* or *DAXX* abnormalities. Subsequent sequencing of *ATRX* and *DAXX* in other cancers revealed *ATRX* mutations in 1.5-14.3% of various tumors of the central nervous system, and these mutations occurred only in tumors exhibiting ALT. Therefore, we concluded that alterations in *ATRX* and *DAXX* are associated with the ALT phenotype in human cancers [6].

In another recent study, we have identified 3 ALT-positive distant metastatic lesions from well-characterized autopsy cases. These cases represent adenocarcinomas arising from the breast, prostate, and pancreas, respectively. While the metastatic lesions were all ALT-positive, the primary cancer focus that other genetic data shows unequivocally gave rise to the metastases, were all ALT-negative. These intriguing data suggest that although not present in the development of the primary tumors, these genomic alterations play an important role in the development of metastatic lesions. This investigation was presented at the special "Telomeres and Telomerase" meeting at the Cold Spring Harbor Laboratories (**Appendix A**). The study was also presented at the 15th Annual Johns Hopkins Department of Pathology Young Investigator's Day as a poster presentation; the trainee was awarded the Excellence in Translational Research Award (**Appendix B**).

To further characterize the ALT phenotype, we developed a novel isogenic cell line pair derived from first generation telomerase knock-out mouse embryonic fibroblasts (MEF). Compared to early-passage telomerase KO cells and late passage WT control cells, late-passage telomerase KO cells spontaneously developed the ALT phenotype. These ALT-positive cells display increased DNA damage, as observed by dramatically increased accumulation of γH2AX foci. These results were presented this year at the AACR Special Meeting: Chromatin and Epigenetics in Cancer (**Appendix C**).

Finally, in collaboration with Dr. Elizabeth Platz (Department of Epidemiology; The Johns Hopkins Bloomberg School of Public Health) and other colleagues at Harvard University, we studied the association between telomere length and progression of prostate cancer - another hormone-dependent tumor type. In this study, we prospectively evaluated the association of telomere length and variability in telomere length among prostate cancer cells and other prostate cell types with risk of prostate cancer outcomes, including prostate cancer death. The men were participants in the large, well-characterized Health Professionals Follow-up Study (HPFS), and consisted of 596 men who were surgically treated for clinically-localized prostate cancer. As described above, we used the telomere-specific FISH assay that provides single cell resolution of telomere length while maintaining tissue architecture to show that men whose prostate cancer cells had more variable telomere length from cell-to-cell and whose prostate cancer-associated stromal cells had shorter telomeres, when in combination, were substantially more likely to progress to metastasis and prostate cancer death than other men. Therefore, this telomere biomarker has the potential to aid in making better treatment and surveillance decisions. These findings were recently accepted for publication and will come out in the October 2013 issue of *Cancer Discovery* (**Appendix D**). Given these exciting results, similar studies for breast cancer, specifically evaluating telomere length variation, have been initiated with Dr. Kala Visvanathan (Department of Epidemiology; The Johns Hopkins Bloomberg School of Public Health).

In addition to the outlined scientific investigations, the trainee has received experimental training in numerous methods including: fluorescence *in situ* hybridization, immunostaining, histopathology, primary cell culture, study design and statistical analysis. The trainee has also interacted and collaborated with oncologists, surgeons, pathologists, molecular epidemiologists and other Ph.D. research scientists who specialize in the research and treatment of breast cancer. The trainee has attended weekly journal clubs, Oncology translational research seminars, breast cancer seminars, Pathology Grand Rounds, specific meetings of the Hopkins Breast SPORE program and "sign-out" sessions with surgical breast pathologists. Finally, the trainee was invited to write a review article describing "***The potential utility of telomere-related markers for cancer diagnosis"*** [7]. In alignment with his stated career goals, he recently submitted an application sponsored by the State of Maryland Cigarette Restitution Fund (CRF) specifically for Faculty Recruitment and Retention at Johns Hopkins (*currently under review*). The title of the application is "***Telomere length alterations as biomarkers for breast and ovarian cancer risk and progression***". The overarching goal of the proposal is to further the understanding of telomere biology in cancer development and progression and to use this knowledge to identify novel biomarkers for the accurate prediction of breast and ovarian cancer risk, early detection, and prognosis.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that dramatic telomere shortening occurs specifically in luminal epithelial cells, but not in myoepithelial cells, in the majority of histologically normal TDLUs from women free of cancer undergoing reduction mammoplasty and in women at time of autopsy without evidence of cancer.

- Demonstrated that the extent and degree of telomere shortening in histologically normal TDLUs varies by the individual.

- Demonstrated that telomere lengths were shorter in the more aggressive breast cancer subtypes, suggesting tumor telomere length may have clinical utility as a prognostic and/or risk stratification biomarker for breast cancer.

- Determined the prevalence of the ALT phenotype in breast carcinoma (2%) and comprehensively surveyed the prevalence of the ALT phenotype in 6,110 primary tumors from a broad range of human cancer subtypes (3.73%).

- Demonstrated that alterations in two genes, *ATRX* and *DAXX,* which participate in chromatin remodeling at telomeres are closely associated with the ALT phenotype in a variety of human cancers.

- Identified 3 ALT-positive distant metastatic lesions from well-characterized autopsy cases (adenocarcinomas arising from the breast, prostate, and pancreas), whereby the primary cancer focus that gave rise to the metastases were all ALT-negative.

- Developed a novel isogenic cell line pair derived from first generation telomerase knock-out (KO) mouse embryonic fibroblasts (MEF), that late-passage telomerase KO cells spontaneously developed the ALT phenotype.

- Observed in a large prospective study that the combination of more variable telomere length among cancer cells and shorter telomere length in cancer-associated stromal cells was strongly associated with progression to metastasis and prostate cancer death.

REPORTABLE OUTCOMES

Peer Reviewed Manuscripts (since last annual report):
C.M. Heaphy, G.S. Yoon, S.B. Peskoe, C.E. Joshu, T.K. Lee, E. Giovannucci, L.A. Mucci, S.A. Kenfield, M.J. Stampfer, J.L. Hicks, A.M. De Marzo, E.A. Platz, and A.K. Meeker. Prostate cancer cell telomere length variability and stromal cell telomere length as prognostic markers for metastasis and death. Cancer Discovery, 2013 June 18. doi:10.1158/2159-8290.CD-13-0135.

Additional Peer Reviewed Manuscripts (during entire funding period):
C.M. Heaphy, A.P. Subhawong, A. Gross, Y. Konishi, N. Kouprina, P. Argani, K. Visvanathan, and A.K. Meeker. Shorter telomeres in luminal B, HER-2 and triple-negative breast cancer subtypes. Modern Pathology, 24:194-200, 2011.

C.M. Heaphy and A.K. Meeker. The potential utility of telomere-related markers for cancer diagnosis. Journal of Cellular and Molecular Medicine, 15:1227-1238, 2011. *Invited review*

C.M. Heaphy, A.P. Subhawong, S-M. Hong, M.G. Goggins, E.A. Montgomery, E. Gabrielson, G.J. Netto, J.I. Epstein, T.L. Lotan, W.H. Westra, I-M. Shih, C.A. Iacobuzio-Donahue, A. Maitra, Q.K. Li, C.G. Eberhart, J.M. Taube, D. Rakheja, R.J. Kurman, T. Wu, R.B. Roden, P. Argani, A.M. De Marzo, L. Terracciano, M. Torbenson and A.K. Meeker. Prevalence of the Alternative Lengthening of Telomeres (ALT) telomere maintenance mechanism in human cancer subtypes. The American Journal of Pathology, 179:1608-1615, 2011.

C.M. Heaphy*, R.F. de Wilde*, Y. Jiao*, A.P. Klein, B.H. Edil, C. Shi, C. Bettegowda, F.J Rodriguez, C.G Eberhart, S. Hebbar, G.J. Offerhaus, R. McLendon, B.A. Rasheed, Y. He, H. Yan, D.D. Bigner, S.M. Oba-Shinjo, S.K. Nagahashi Marie, G.J. Riggins, K.W. Kinzler, B. Vogelstein, R.H. Hruban, A. Maitra, N. Papadopoulos and A.K. Meeker. Altered telomeres in tumors with *ATRX* and *DAXX* mutations. Science, 333:425, 2011.
**Authors contributed equally to this study*

Published Abstracts at National Meetings (since last annual report):
C.M. Heaphy, M.C. Haffner, P. Argani, C.A. Iacobuzio-Donahue, V. Yegnasubramanian and A.K. Meeker (2013). The ALT phenotype with associated ATRX/DAXX alterations define a novel molecular subclass of lethal metastatic disease. Cold Spring Harbor Laboratory Meeting – Telomeres and Telomerase. Cold Spring Harbor, NY.

C.M. Heaphy, B.A. Poore, M.C. Haffner, and A.K. Meeker (2013). A novel cell line model of the Alternative Lengthening of Telomeres (ALT) telomere maintenance mechanism. AACR Special Meeting: Chromatin and Epigenetics in Cancer. Atlanta, GA

Additional Published Abstracts at National Meetings (during entire funding period):
C.M. Heaphy, A.P. Subhawong, S. Hong, M. Goggins, E. Montgomery, E. Gabrielson, G. Netto, J.I. Epstein, T.L. Lotan, W. Westra, I. Shih, C. Iacobuzio-Donahue, A. Maitra, Q. Li, C. Eberhart, J. Taube, R. Kurman, T Wu, R. Roden, P. Argani, A.M. De Marzo, L. Terracciano, M. Torbenson and A.K. Meeker (2011). Prevalence of Alternative Lengthening of Telomeres (ALT) in human cancer subtypes. United States and Canadian Academy of Pathology (USCAP) Annual Meeting. San Antonio, TX.

C.M. Heaphy, M.E. Sherman, B.K. Vonderhaar, P. Argani and A.K. Meeker (2011). Cellular Consequences of Telomere Shortening in Histologically Normal Breast Tissues. Era of Hope DoD Conference, Orlando, FL.

C.M. Heaphy, M.E. Sherman, B.K. Vonderhaar, P. Argani and A.K. Meeker (2011). Significant telomere shortening is common in luminal epithelial cells in histologically normal breast tissues from women without cancer. AACR Special Meeting: Advances in Breast Cancer Research. San Francisco, CA.

Awards (since last annual report):
The 15th Annual Johns Hopkins Department of Pathology Young Investigator's Day Award for Excellence in Translational Research for poster presentation titled "The ALT phenotype with associated ATRX/DAXX alterations define a novel molecular subclass of lethal metastatic disease" (May 2013).

Additional Awards (during entire funding period):
Stowell-Orbison Award at the United States and Canadian Academy of Pathology (USCAP) Annual Meeting for poster presentation titled "Prevalence of Alternative Lengthening of Telomeres (ALT) in Human Cancer Subtypes" (April 2011).

The 13th Annual Johns Hopkins Department of Pathology Young Investigator's Day Award for Excellence in Translational Research for poster presentation titled "Prevalence of Alternative Lengthening of Telomeres (ALT) in Human Cancer Subtypes" (May 2011).

1st Place for Basic Research in the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins Fellow Research Day for a poster presentation titled "Altered telomeres in tumors with *ATRX* and *DAXX* mutations" (May, 2012).

CONCLUSIONS

Through this training grant, data generated have been have been presented at numerous national meetings. Importantly, the postdoctoral trainee is a first author on a number of manuscripts published in high-profile journals (eg. ***Science***, ***The American Journal of Pathology, Cancer Discovery***). Another manuscript was published in ***Modern Pathology***; and, an invited review article assessing the potential utility of telomere-related markers in the field of cancer diagnosis

was published in the ***Journal of Cellular and Molecular Medicine***. The investigator has fulfilled all his educational and training goals.

REFERENCES

1. Speirs V, Green AR, Walton DS, Kerin MJ, Fox JN, Carleton PJ, Desai SB, Atkin SL. Short-term primary culture of epithelial cells derived from human breast tumours. *Br J Cancer*. 1998; 78: 1421-9.

2. Heaphy CM, Subhawong AP, Gross AL, Konishi Y, Kouprina N, Argani P, Visvanathan K, Meeker AK. Shorter telomeres in luminal B, HER-2 and triple-negative breast cancer subtypes. *Mod Pathol*. 2011; 24: 194-200.

3. Subhawong AP, Heaphy CM, Argani P, Konishi Y, Kouprina N, Nassar H, Vang R, Meeker AK. The alternative lengthening of telomeres phenotype in breast carcinoma is associated with HER-2 overexpression. *Mod Pathol*. 2009; 22: 1423-31.

4. Heaphy CM, Subhawong AP, Hong SM, Goggins MG, Montgomery EA, Gabrielson E, Netto GJ, Epstein JI, Lotan TL, Westra WH, Shih Ie M, Iacobuzio-Donahue CA, Maitra A, Li QK, Eberhart CG, Taube JM, Rakheja D, Kurman RJ, Wu TC, Roden RB, Argani P, De Marzo AM, Terracciano L, Torbenson M, Meeker AK. Prevalence of the alternative lengthening of telomeres telomere maintenance mechanism in human cancer subtypes. *Am J Pathol*. 2011; 179: 1608-15.

5. Jiao Y, Shi C, Edil BH, de Wilde RF, Klimstra DS, Maitra A, Schulick RD, Tang LH, Wolfgang CL, Choti MA, Velculescu VE, Diaz LA, Jr., Vogelstein B, Kinzler KW, Hruban RH, Papadopoulos N. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. *Science*. 2011; 331: 1199-203.

6. Heaphy CM, de Wilde RF, Jiao Y, Klein AP, Edil BH, Shi C, Bettegowda C, Rodriguez FJ, Eberhart CG, Hebbar S, Offerhaus GJ, McLendon R, Rasheed BA, He Y, Yan H, Bigner DD, Oba-Shinjo SM, Marie SK, Riggins GJ, Kinzler KW, Vogelstein B, Hruban RH, Maitra A, Papadopoulos N, Meeker AK. Altered telomeres in tumors with ATRX and DAXX mutations. *Science*. 2011; 333: 425.

7. Heaphy CM, Meeker AK. The potential utility of telomere-related markers for cancer diagnosis. *J Cell Mol Med*. 2011; 15: 1227-38.

The ALT phenotype with associated ATRX/DAXX alterations define a novel molecular subclass of lethal metastatic disease

Christopher M Heaphy[1], Michael C Haffner[2], Pedram Argani[1,2], Christine A Iacobuzio-Donahue[1,2], Vasan Yegnasubramanian[2], Alan K Meeker[1,2]

[1]Johns Hopkins University School of Medicine, Pathology, Baltimore, MD, 21231, [2]Johns Hopkins University, Oncology, Baltimore, MD, 21231

Unlimited replicative capacity is a key hallmark of cancer that requires the abrogation of telomere attrition due to the end-replication problem. The vast majority of tumors maintain telomere lengths by expressing the enzyme telomerase; however, a subset utilizes a cancer-specific, telomerase-independent telomere maintenance mechanism, termed Alternative Lengthening of Telomeres (ALT). Recent sequencing efforts have highlighted the importance of identifying specific genomic signatures that may be targeted with personalized treatment strategies. To this end, we recently uncovered a strong correlation between ALT-positive primary tumors and recurrent cancer-associated somatic inactivating mutations in the *ATRX* and *DAXX* genes. In addition, using a robust telomere-specific fluorescent *in situ* hybridization assay for detecting ALT in standard fixed tissue specimens, we surveyed more than 6100 primary tumors from 94 different cancer subtypes. We confirmed that ALT is present in a small subset of breast carcinomas (2%; N=251), but surprisingly did not detect even a single case of ALT in primary adenocarcinomas arising from the prostate (N=1152) or pancreas (N=448). Interestingly, we have recently identified 3 ALT-positive distant metastatic lesions from well-characterized autopsy cases. These cases represent adenocarcinomas arising from the breast, prostate, and pancreas, respectively. For the prostatic and pancreatic cases where genomic data was available, mutations in *ATRX* were identified in the metastatic lesions, but were not present in the primary cancer focus that other genetic data shows unequivocally gave rise to the metastases. Furthermore, recently published exomic sequencing has shown that *ATRX* and *DAXX* are altered in a fraction of advanced castration-resistant prostate cancers; thus supporting the hypothesis that this molecular signature (ALT with an altered ATRX/DAXX complex) defines a substantial fraction of lethal, metastatic prostate cancer. These intriguing data suggest that although not present in the development of the primary tumors, these genomic alterations play an important role in the development of metastatic lesions. Therefore, understanding the biology of these particular metastases will provide new therapeutic targets for late stage disease.

TITLE: The ALT phenotype with associated ATRX/DAXX alterations define a novel molecular subclass of lethal metastatic disease

AUTHORS: Christopher M Heaphy[1], Michael C Haffner[2], Pedram Argani[1,2], Christine A Iacobuzio-Donahue[1,2], Vasan Yegnasubramanian[2], Alan K Meeker[1,2]

[1]Johns Hopkins University School of Medicine, Pathology, Baltimore, MD, [2]Johns Hopkins University School of Medicine, Oncology, Baltimore, MD

Unlimited replicative capacity is a key hallmark of cancer that requires the abrogation of telomere attrition due to the end-replication problem. The vast majority of tumors maintain telomere lengths by expressing the enzyme telomerase; however, a subset utilizes a cancer-specific, telomerase-independent telomere maintenance mechanism, termed Alternative Lengthening of Telomeres (ALT). Recent sequencing efforts have highlighted the importance of identifying specific genomic signatures that may be targeted with personalized treatment strategies. To this end, we recently uncovered a strong correlation between ALT-positive primary tumors and recurrent cancer-associated somatic inactivating mutations in the *ATRX* and *DAXX* genes. In addition, using a robust telomere-specific fluorescent *in situ* hybridization assay for detecting ALT in standard fixed tissue specimens, we surveyed more than 6100 primary tumors from 94 different cancer subtypes. We confirmed that ALT is present in a small subset of breast carcinomas (2%; N=251), but surprisingly did not detect even a single case of ALT in primary adenocarcinomas arising from the prostate (N=1152) or pancreas (N=448). Interestingly, we have recently identified 3 ALT-positive distant metastatic lesions from well-characterized autopsy cases. These cases represent adenocarcinomas arising from the breast, prostate, and pancreas, respectively. For the prostatic and pancreatic cases where genomic data was available, mutations in *ATRX* were identified in the metastatic lesions, but were not present in the primary cancer focus that other genetic data shows unequivocally gave rise to the metastases. Furthermore, recently published exomic sequencing has shown that *ATRX* and *DAXX* are altered in a fraction of advanced castration-resistant prostate cancers; thus supporting the hypothesis that this molecular signature (ALT with an altered ATRX/DAXX complex) defines a substantial fraction of lethal, metastatic prostate cancer. These intriguing data suggest that although not present in the development of the primary tumors, these genomic alterations play an important role in the development of metastatic lesions. Therefore, understanding the biology of these particular metastases will provide new therapeutic targets for late stage disease.

A novel cell line model of the Alternative Lengthening of Telomeres (ALT) telomere maintenance mechanism

Christopher M Heaphy[1], Michael C Haffner[2], Alan K Meeker[1,2]

[1]Johns Hopkins University School of Medicine, Pathology, Baltimore, MD, 21231, [2]Johns Hopkins University School of Medicine, Oncology, Baltimore, MD, 21231

Unlimited replicative capacity is a key hallmark of cancer that requires the abrogation of telomere attrition due to the end-replication problem. The vast majority of tumors maintain telomere lengths by expressing the enzyme telomerase; however, a subset utilizes a cancer-specific, telomerase-independent telomere maintenance mechanism, termed Alternative Lengthening of Telomeres (ALT). We recently uncovered a strong correlation between ALT and recurrent cancer-associated somatic inactivating mutations in the *ATRX* and *DAXX*. The ATRX and DAXX proteins form a chromatin remodeling complex with histone chaperone activity, targeting incorporation of the histone variant H3.3 to G-rich repetitive regions of the genome, including the telomeres, which are known to normally be maintained in a repressed heterochromatic state. Here, we have developed an isogenic cell line pair derived from first generation telomerase knock-out mouse embryonic fibroblasts (MEF). Compared to early-passage telomerase KO cells and late passage WT control cells, late-passage telomerase KO cells spontaneously developed the ALT phenotype. These ALT(+) cells display increased DNA damage, as observed by dramatically increased accumulation of γH2AX foci. We plan to use this unique cell line to identify drugs exhibiting selective toxicity for ALT(+) cells. Preliminary results indicate that ALT(+) late-passage telomerase KO cells exhibit an increased sensitivity to the DNA-PK inhibitor NU7441 compared to the isogenic early-passage ALT(-) telomerase KO cells. The identification of *bona fide* ALT-specific drugs will pave the way for the development of new targeted treatments for the significant number of cancers that harbor this unique molecular phenotype.

CANCER DISCOVERY

Prostate Cancer Cell Telomere Length Variability and Stromal Cell Telomere Length as Prognostic Markers for Metastasis and Death

Christopher M Heaphy, Ghil Suk Yoon, Sarah B Peskoe, et al.

Cancer Discovery Published OnlineFirst June 18, 2013.

Prostate Cancer Cell Telomere Length Variability and Stromal Cell Telomere Length as Prognostic Markers for Metastasis and Death

Christopher M. Heaphy,[1]* Ghil Suk Yoon,[1,2]* Sarah B. Peskoe,[3] Corinne E. Joshu,[3] Thomas K. Lee,[1] Edward Giovannucci,[4,5,6] Lorelei A. Mucci,[5,6] Stacey A. Kenfield,[5,6] Meir J. Stampfer,[4,5,6] Jessica L. Hicks,[1] Angelo M. De Marzo,[1,7,8] Elizabeth A. Platz,[3,7,8]**† Alan K. Meeker[1,7,8]**†

*These authors contributed equally to this study

**These authors contributed equally to this study

[1]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD
[2]Department of Pathology, Kyungpook National University School of Medicine, Daegu, Korea
[3]Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
[4]Department of Nutrition, Harvard School of Public Health, Boston, MA
[5]Department of Epidemiology, Harvard School of Public Health, Boston, MA
[6]Department of Medicine, Channing Division of Network Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA
[7]James Buchanan Brady Urological Institute, Johns Hopkins School of Medicine, Baltimore, MD
[8]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD

†To whom correspondence should be addressed.

Elizabeth A. Platz, ScD, MPH
Professor
Department of Epidemiology
John Hopkins Bloomberg School of Public Health
615 N. Wolfe Street, Rm E6132
Baltimore, MD 21205
Email: eplatz@jhsph.edu

Alan K. Meeker, PhD
Assistant Professor
Department of Pathology
Johns Hopkins School of Medicine
411 N. Caroline St, Bond Street B301
Baltimore, MD 21231
Email: ameeker1@jhmi.edu

Running title: Telomere length as a prognostic marker for prostate cancer

Keywords: Telomere, prostate cancer, prognostic marker

Disclosures: Dr. Angelo M. De Marzo is currently employed at Predictive Biosciences, Inc., Lexington MA, as well being part-time adjunct Professor of Pathology, Oncology, and Urology at the Johns Hopkins University School of Medicine. No funding or other support was provided by Predictive Biosciences, Inc. for any of the work in this manuscript. The terms of this relationship are managed by the Johns Hopkins University in accordance with its conflict-of-interest policies. There are no other disclosures.

Text word count: 6,279

Total number of figures and tables: 2 figures, 4 tables

STATEMENT OF SIGNIFICANCE

In this prospective study, the combination of more variable telomere length among cancer cells and shorter telomere length in cancer-associated stromal cells was strongly associated with progression to metastasis and prostate cancer death, pointing to the translational potential for prognostication and risk stratification for individualized therapeutic and surveillance strategies.

ABSTRACT

Current prognostic indicators are imperfect predictors of outcome in men with clinically-localized prostate cancer. Thus, tissue-based markers are urgently needed to improve treatment and surveillance decision-making. Given that shortened telomeres enhance chromosomal instability and such instability is a hallmark of metastatic lesions, we hypothesized that alterations in telomere length in the primary cancer would predict risk of progression to metastasis and prostate cancer death. To test this hypothesis, we conducted a prospective cohort study of 596 surgically treated men who participated in the ongoing Health Professionals Follow-up Study. Men who had the combination of more variable telomere length among prostate cancer cells (cell-to-cell) and shorter telomere length in prostate cancer-associated stromal cells were substantially more likely to progress to metastasis or die of their prostate cancer. These findings point to the translational potential of this telomere biomarker for prognostication and risk stratification for individualized therapeutic and surveillance strategies.

INTRODUCTION

Currently used prognostic indicators inadequately predict prostate cancer behavior, particularly in men with clinically-localized disease. To target men with appropriate, individualized treatment strategies and surveillance, new molecular markers that improve prognostic accuracy beyond the currently used indicators – stage, Gleason sum and PSA concentration at diagnosis – are urgently needed.

One such possible molecular marker is telomere length. Telomeres are specialized nucleoprotein structures that are essential for maintaining chromosomal integrity by protecting the ends of chromosomes from degradation and recombination (*1-3*). Critical telomere shortening is a common abnormality observed early in prostate tumorigenesis, where it likely helps drive malignant transformation and tumor progression via telomere destabilization and concomitant chromosomal instability (*4*).

Preliminary investigations have suggested that reduced telomere length in prostate cancer tissue may be associated with poor clinical outcome in prostate cancer (*5-7*). While intriguing, these studies used bulk measures of tissue telomere length, and without individual cell resolution, could not address associations with outcome for telomere length in specific cells types or for variability in telomere length from cell-to-cell. Some have suggested measures of genetic or phenotypic variability or diversity at the cellular level may prove to be useful prognostic biomarkers of cancer behavior (*8*).

Thus, we prospectively evaluated the association of telomere length and variability in telomere length among prostate cancer cells and other prostate cell types with risk of prostate cancer outcomes, including prostate cancer death, and also non-prostate cancer death in 596 men who were surgically treated for clinically-localized

prostate cancer; the men were participants in the large, well-characterized Health Professionals Follow-up Study (HPFS) (https://www.hsph.harvard.edu/hpfs). Using a telomere-specific fluorescence *in situ* hybridization (FISH) assay that provides single cell resolution of telomere length while maintaining tissue architecture, we report below that men whose prostate cancer cells had more variable telomere length from cell to cell and whose prostate cancer-associated stromal (CAS) cells had shorter telomeres, when in combination, were substantially more likely to progress to metastasis and prostate cancer death than other men. Notably, these findings for the telomere biomarker were independent of currently used prognostic indicators, including in men with intermediate risk disease. If confirmed, the biomarker has the potential to aid in making better treatment and surveillance decisions.

RESULTS

Characteristics of the men

The men were 65.3 years old on average at diagnosis, the majority were white, had a prostatectomy Gleason sum of 7 (3+4 or 4+3), had pathologically organ-confined disease, and of those for whom PSA concentration at diagnosis was available, had a PSA concentration <10 ng/mL **(Table 1)**. The mean follow-up times were 10.7 years for biochemical recurrence, 13.1 years for lethal prostate cancer (either progression to distant metastasis or prostate cancer death), and 13.2 years for prostate cancer death and for non-prostate cancer death. The Kaplan-Meier estimates of the cumulative incidences over follow-up were: biochemical recurrence 33%, lethal prostate cancer 19%, prostate cancer death 17%, and non-prostate cancer death 56%, all over a maximum follow-up of 23 years. Given the men's characteristics and rates of recurrence, this cohort is relevant to men in the PSA era diagnosed with clinically-localized disease.

Telomere FISH staining provides single cell resolution, allowing high-resolution assessment of telomere length and variability in length from cell to cell

Telomere-specific FISH signals are linearly proportional to telomere length and thus, telomere length can be quantified via digital image analysis (9). As expected, signals were less intense (i.e., telomere length was shorter) in cancer cells, on average, than in adjacent cells in prostate tissue samples from the men in the HPFS. **Figure 1** shows representative examples of telomere signals for individual cells. For some men, telomere signals were variable in intensity from cancer cell to cancer cell **(Fig. 1A)**. For

other men, telomere signals were less variable in intensity; **Fig. 1B** shows an example where telomere signals were uniformly diminished in cancer cells. We also observed that telomere signals were decreased in CAS cells in some of the men **(Fig. 1C)** compared with other men **(Fig. 1D)**.

Shorter telomeres in prostate CAS cells, and more variable telomere length among prostate cancer cells are associated with increased risk of poor prostate cancer outcomes

We assessed telomere length, on a per cell basis, as the ratio of the total intensity of telomeric signals in each cell to the total intensity of the DAPI stained nuclear DNA signal in the same cell (see **Methods**)**.** Then, we examined the association of median telomere length and the standard deviation of telomere length (as a measure of cell-to-cell variability), which we calculated for each man separately by cell type, with prostate cancer outcomes and with non-prostate cancer death after adjusting for commonly used prognostic indicators.

Compared with the longest tertile, the shortest and middle tertiles of median telomere length in CAS cells had a statistically significant increased risk of lethal prostate cancer (shortest: hazard ratio [HR]=2.42, 95% CI 1.16-5.07; middle: HR=2.44, 95% CI 1.17-5.11; *P*-trend=0.02) and prostate cancer death (shortest: HR=2.85, 95% CI 1.22-6.69; middle: HR=3.02, 95% CI 1.31-6.97; *P*-trend=0.02), but not of biochemical recurrence or non-prostate cancer death. Median telomere length in the other cell types assessed (cancer, cancer-associated luminal epithelial, cancer-associated basal epithelial, normal luminal epithelial, normal basal epithelial, and normal stromal) was not

associated with the outcomes. Considering telomere length variability, compared with the least variable tertile, the most variable tertile among prostate cancer cells had a higher risk of biochemical recurrence (*P*-trend=0.01), and while not statistically significant, the HR for the most variable tertile compared with the least variable tertile was above 1.0 for both lethal prostate cancer (HR=1.39, 95% CI 0.73-2.67; *P*-trend=0.17) and prostate cancer death (HR=1.52, 95% CI 0.73-3.18; *P*-trend=0.12). This pattern was also evident for non-prostate cancer death (primarily death due to cardiovascular diseases and other cancers; HR=1.26, 95% CI 0.83-1.92; *P*-trend=0.23). Risk was similar when comparing the middle and least variable tertiles among cancer cells for all four outcomes. None of the other cell types assessed had a consistent pattern of association for telomere length variability. We also evaluated the association for telomere length variability from cancer cell to cancer cell using nonparametric measures and the inferences were comparable. The coefficient of variation (CV%; i.e., standard deviation / mean) for telomere length among the cancer cells was not associated with the outcomes.

Based on these findings, we focused on cancer cells and CAS cells for the subsequent analyses. We calculated telomere length and variability in telomere length among the cancer cells and in the CAS cells, and explored the relationship between length and variability in length within and between the two cell types. Cancer cells (median=12.9 [ratio of the total intensity of telomeric signals to the total intensity of the DAPI signal]) had substantially shorter telomere length than CAS cells (median=55.8). Cancer cells had a smaller standard deviation for telomere length, but when standardized for the mean length (i.e., the CV%), the relative variability was greater

among cancer cells (standard deviation=8.4, CV%=58.1%) than in CAS cells (standard deviation=24.6, CV%=44.4%). Median telomere length in CAS cells increased across tertiles of variability in telomere length (least to most variable tertiles: 43.4, 56.3, 66.7). Variability in telomere length among the prostate cancer cells increased across tertiles of median telomere length in CAS cells (shortest to longest tertiles: 6.4, 8.1, 11.0). For cancer cells, the midpoint of the most variable tertile was 3.6 times larger than the midpoint of the least variable tertile. For the CAS cells, the midpoint of the longest tertile of median telomere length was 2.5 times larger than the midpoint of the shortest tertile of median telomere length, indicating a wide dynamic range in both telomere length and variability in length.

Because the HRs of lethal prostate cancer for the shortest and middle tertiles of median telomere length in CAS cells were similar, we combined those tertiles to form a single “shorter” length group. Likewise, because the HRs of lethal prostate cancer for the least and middle tertiles of variability in telomere length among prostate cancer cells were similar, we combined those tertiles to form a single “less variable” length group.

Prostate cancer cell-to-cell variability in telomere length and CAS cell telomere length are associated with prognostic indicators

We next determined how variability in telomere length among cancer cells and telomere length in CAS cells relate to currently used prognostic indicators. Median variability in telomere length from cancer cell to cancer cell increased with increasing prostatectomy Gleason sum (*P*-trend=0.0002) and was higher in T3b or worse disease (*P*=0.05), but did not notably increase with increasing PSA concentration at diagnosis (*P*-trend=0.45).

Median telomere length in CAS cells did not differ across prostatectomy Gleason sum (*P*-trend=0.38), pathologic stage (*P*=0.60), or PSA concentration at diagnosis (*P*-trend=0.11).

More variable telomere length among prostate cancer cells and shorter telomeres in CAS cells are associated with an increased risk of poor prostate cancer outcomes independent of prognostic indicators

Shown in **Table 2** are the associations for more versus less variable telomere length among cancer cells and shorter versus longer telomere length in CAS cells for each outcome after a) adjustment for age and year of diagnosis, b) further adjustment for prognostic indicators, and c) because the median telomere length in the CAS cells was related to the variability in telomere length among the cancer cells and vice versa, even further adjustment for shorter length in the variability analysis and for more variability in the shorter length analysis. For lethal prostate cancer and for prostate cancer death, both more variable telomere length among cancer cells and shorter telomeres in CAS cells were associated with increased risk. The association for variability in telomere length among cancer cells was attenuated after adjustment for the prognostic indicators, but was strengthened and was significant after further adjustment for shorter telomeres in CAS cells. The association for shorter telomeres in CAS cells was strengthened after adjustment for the prognostic indicators and even further strengthened after adjustment for variability in telomere length among cancer cells. For biochemical recurrence, more variable telomere length among cancer cells was associated with increased risk even after adjustment for the prognostic indicators, while shorter telomeres in CAS cells was

not associated with risk. For non-prostate cancer death, variability in telomere length among cancer cells was not statistically significantly associated with risk, although shorter telomere length in CAS cells was possibly, but not statistically significantly, inversely associated.

Defining the "the telomere biomarker" and its association with prognostic indicators

Given that the associations of more variable telomere length from cancer cell to cancer cell and shorter telomeres in CAS cells with prostate cancer outcomes were independent of one another, we combined them to form four groups: less variable [among cancer cells]/longer [in CAS cells] (reference), more variable/longer, less variable/shorter, and more variable/shorter. We refer to the combined groups as "the telomere biomarker". Prostatectomy Gleason sum, pathologic stage, and PSA concentration at diagnosis differed among the telomere biomarker groups **(Table 1)**. The less variable/longer combination tended to have the most favorable clinicopathologic prognostic indicators, the more variable/shorter and more variable/longer combinations tended to have similar and less favorable indicators, and the less variable/shorter combination had indicators that were intermediate **(Table 1)**.

Men with the more variable/shorter combination of the telomere biomarker had poorer disease-free survival than other men

Figure 2 shows survival curves for the four telomere biomarker groups; these results are unadjusted. For biochemical recurrence **(Fig. 2A)**, men with the more

variable/shorter and more variable/longer combinations had similarly higher risk over time, men with the less variable/longer combination had the lowest risk, and men with the less variable/shorter combination had an intermediate risk (overall log-rank test comparing the 4 combinations: P=0.002). Men with the more variable/shorter combination were the most likely to experience lethal prostate cancer **(Fig. 2B)** and prostate cancer death **(Fig. 2C)** over time, whereas the men with the less variable/longer combination were the least likely to experience these outcomes, and men with the other two combinations had intermediate risk (for both outcomes - logrank test comparing the 4 combinations: P<0.0001; logrank test comparing the more variable/shorter to less variable/longer: P<0.0001). The telomere biomarker groups did not statistically significantly differ (P=0.20) on risk of non-prostate cancer death over time **(Fig. 2D)**, supporting the specificity of the biomarker for prostate-cancer outcomes.

The more variable/shorter combination of the telomere biomarker is strongly associated with increased risk of poor prostate cancer outcomes independent of prognostic indicators

Table 3 shows the association between each telomere biomarker group and outcomes after a) adjustment for age and year of diagnosis, and b) further adjustment for the commonly used prognostic indicators. Men with the more variable/shorter combination were more likely to subsequently progress to lethal prostate cancer and prostate cancer death after adjustment for age and year of diagnosis, and even after further taking the prognostic indicators into account. When compared with men with the less variable/longer combination, men with the more variable/shorter combination had 8

times the risk of lethal prostate cancer (*P*=0.005) and 14 times the risk of prostate cancer death (*P*=0.01) after multivariable adjustment. Men with the other two combinations possibly had a higher risk of lethal prostate cancer and prostate cancer death, although the results were not statistically significant. The more variable/shorter combination was weakly associated with biochemical recurrence. The telomere biomarker was not associated with non-prostate cancer death. Taken together, these results support the potential utility of the telomere biomarker as a prognostic indicator specifically of poor prostate cancer outcome.

Men with the less variable/longer combination of the telomere biomarker were much less likely to die of their prostate cancer than expected, and their time to poor outcome was much longer

Only 1 man (expected 5.8 men) in the less variable/longer combination died of his prostate cancer, whereas 20 men (expected 10.4 men) in the more variable/shorter combination died of their prostate cancers. The time from diagnosis to prostate cancer death was 16.5 years for the former man despite having Gleason 9 (stage T2aN0M0) disease, whereas the median time from diagnosis to prostate cancer death was 8.4 years for the latter men. Adjusting for the prognostic indicators, men with the less variable/longer combination had an HR of lethal prostate cancer of 0.23 (95% CI 0.06-0.93; *P*=0.04) and an HR of prostate cancer death of 0.13 (95% CI 0.02-0.96; *P*=0.05) when compared to men with all other telomere biomarker combinations. These findings support that the telomere biomarker may point to men who are unlikely to progress or who may progress more slowly after surgical treatment.

The telomere biomarker adds to the predictive capability of the currently used prognostic indicators

We considered the predictive capability of the telomere biomarker for prostate cancer outcomes relative to the currently used prognostic indicators. In the multivariable model that included the prognostic indicators plus the telomere biomarker, the HRs of lethal prostate cancer and prostate cancer death for the more variable/shorter combination were of the same order of magnitude and statistical significance as for prostatectomy Gleason sum and pathologic stage **(Table 4)**. We also calculated the C-statistic (i.e., the area under the receiver operating characteristics curve) for the telomere biomarker. For lethal prostate cancer, the C-statistic improved from 0.63 to 0.74 when adding the telomere biomarker to the model with age and year of diagnosis. When adding the telomere biomarker to the model with the prognostic indicators stage, prostatectomy Gleason sum, and PSA concentration at diagnosis, the C-statistic improved from 0.85 to 0.87. For prostate cancer death, the C-statistic improved from 0.67 to 0.79 when adding the telomere biomarker to the model with age and year of diagnosis. When adding the telomere biomarker to the model with the prognostic indicators, the C-statistic improved from 0.91 to 0.93. Thus, we have documented that the telomere biomarker is an independent predictor of poor outcome, and it has the potential to add to the predictive capability of the currently used prognostic indicators.

Notably, the telomere biomarker predicts poor outcome even in men with intermediate risk disease

Treatment decision-making for clinically-localized Gleason 7 prostate cancer is difficult because of variable disease course. Thus, we determined whether the telomere biomarker improves prognostication for these men (N=351). When comparing the more variable/shorter combination to all other combinations, the HR of lethal prostate cancer was 3.67 (95% CI 1.60-8.40, *P*=0.002) in men with Gleason 7 disease (in all men: HR=2.83, 95% CI 1.59-5.03, *P*=0.0004). For prostate cancer death, the HR was 7.13 (95% CI 2.71-18.77; *P*<0.0001) in men with Gleason 7 disease (in all men: HR=3.12, 95% CI 1.68-5.77, *P*=0.0003). Further, among men with Gleason 7 disease, when adding the telomere biomarker to the currently used prognostic indicators, the C-statistic improved from 0.82 to 0.84 for lethal prostate cancer, and from 0.85 to 0.90 for prostate cancer death. Importantly, even in intermediate risk disease, the telomere biomarker may identify men who are more or less likely to experience poor outcome.

DISCUSSION

In this prospective study, men with more variable telomere length from prostate cancer cell to prostate cancer cell and shorter telomeres in prostate cancer-associated stromal cells had 8 times the risk of progressing to lethal prostate cancer, and 14 times the risk of dying of their prostate cancer when compared with men with less variable telomere length among prostate cancer cells and longer telomeres in prostate-cancer associated stromal cells. These associations were independent of the currently used prognostic indicators. In contrast, men who had less variable telomere length from cancer cell to cancer cell and had longer telomeres in CAS cells were 87% less likely to die of their prostate cancers. The telomere biomarker added to the capability of the currently used prognostic indicators for predicting poor outcome in men surgically treated for clinically-localized prostate cancer, even in men with intermediate risk disease. The excess of prostate cancer deaths in the more variable/shorter combination and the deficit of deaths in the less variable/longer combination suggest that the telomere biomarker may have utility in identifying men who may and may not require additional treatment and enhanced surveillance.

Telomeres are comprised of the repeating hexanucleotide DNA sequence, TTAGGG, bound by the six-member shelterin protein complex (*1, 2*). This telomere complex maintains chromosomal stability by inhibiting exonucleolytic degradation, inhibiting inappropriate homologous recombination, and preventing the chromosome ends from being recognized as double-strand breaks, thereby averting chromosomal fusions (3, 10). In normal somatic cells, critical telomere shortening leads to p53-dependent senescence or apoptosis (11, 12). In cancer cells, cell cycle checkpoints are

typically abrogated. In this setting, critical telomere shortening and chromosomal breakage-fusion-bridge cycles may lead to genomic instability (13). Using high-resolution *in situ* methods, extensive telomere shortening has been observed in cancer cells compared with normal epithelial cells in the vast majority of prostate cancers and in high-grade prostatic intraepithelial neoplasia (4, 14).

Given that dysfunctional telomeres contribute to genomic instability and promotes tumorigenesis (15), we hypothesized that increased telomere shortening in prostate cancer cells would drive the evolution of cell clones capable of invasion, extravasation, and metastasis. Therefore, we expected that prostate cancers possessing the greatest degree of telomere loss would have a more aggressive disease phenotype and thus a worse outcome. While we verified that telomeres were shorter, on average, in cancer cells than in neighboring benign-appearing cells, we found that *variability* in telomere length among the cancer cells, rather than telomere length, was associated with risk of poor outcome. Shorter telomeres in cancer-associated stromal cells were even more strongly associated with risk of poor outcome. The combination of variability in telomere length among cancer cells and telomere length in cancer-associated stromal cells, which we call the “telomere biomarker”, was a stronger predictor of prostate cancer outcome than either alone. Notably, the telomere biomarker was also strongly associated and predictive of outcome in men with Gleason 7 disease.

Only three studies have investigated telomere length and prostate cancer outcomes previously; these were small retrospective studies (*5*-*7*) that used DNA extracted from cancer-containing tissue sections. These studies observed statistically significant independent associations of reduced telomeric DNA content, reflecting

shorter telomeres, in prostatectomy specimens (*5, 7*) and in biopsy specimens (*6*) with risk of prostate cancer recurrence or death. In contrast to those studies, our study was prospective, 5-times larger, and our method provided individual cell resolution, thus enabling us to evaluate the contributions of telomere length differences in specific malignant and benign-appearing cell types to clinical outcome.

Recent studies have observed telomere shortening in cancer-associated stromal cells (16, 17); such shortening may reflect a microenvironment that promotes tumor progression or may be a consequence of the tumor on surrounding cells (16). Regarding the former possibility, mounting evidence suggests that microenvironmental alterations may initiate and promote prostate carcinogenesis. During normal development, stromal cells profoundly influence epithelial differentiation. In prostate tumors, the cancer-associated stroma frequently displays an altered gene expression profile (18, 19) and an increase in myofibroblasts and fibroblasts mimicking wound repair, a phenotype known as “reactive stroma” (20). Cunha and colleagues have demonstrated that prostate cancer-associated fibroblasts can induce proliferation and malignant transformation of cultured benign prostate epithelial cells (21). The prostate cancer-associated stroma can help promote tumor progression via several mechanisms including the expression of pro-tumorigenic factors (22). Relevant to our finding of an increased risk of poor outcome in men with shorter stromal telomeres, telomere shortening in fibroblasts has been shown to lead to a senescent phenotype that includes an altered pattern of secreted factors, many of which are known to be tumor promoting, including pro-inflammatory cytokines and matrix-degrading proteases (23). How might the stromal cells develop telomere shortening? While speculative, these

reactive stromal cells may be developing telomere shortening as a response to tissue injury caused by the tumor cells. How might the tumor develop increased telomere length variability from cell to cell? Perhaps this variability reflects or leads to more generalized genetic instability, which in multiple cancer types tends to be related to more aggressive features (24). Future studies in which whole genome sequencing is employed in cases with and without high variability in telomere length from cancer cell to cancer cell could help answer this question.

Several aspects of our study merit discussion. With respect to generalizability, the men we studied are highly relevant to men who are being diagnosed with clinically-localized disease today. While the majority of men in the study were white, reflecting the demographics of the men who entered the health professions during a prior era, we do not have any evidence that our findings would not also apply to men of other racial/ethnic backgrounds. We selected the largest and usually the highest Gleason sum tumor focus then sampled multiple regions of that tumor focus selected to capture within-tumor heterogeneity. Given our tumor sampling strategy, we could not determine whether the telomere biomarker had different predictive capability by tumor focus in men with multiple foci. We used a method of telomere length determination that we previously documented to be both valid and reliable (9). For the assessment of telomere length we evaluated each cell type, where available. For some men, the tissue microarray (TMA) spots, which were sampled from areas of adenocarcinoma, did not contain sufficient normal-appearing luminal epithelial, basal epithelial, or stromal cells for analysis. Thus, the number of men in those analyses was smaller than for the cancer cell analysis. Cells of each type that were in sharp focus in the digital image of the TMA

spots were selected for telomere length determination, but otherwise were not sampled with respect to the appearance of the cells. Nevertheless, the evaluated cells were not a random sample and it was not feasible to evaluate all potentially evaluable cells because the assay in its current implementation is extremely labor intensive. We used the ratio of the total intensity of telomeric signals to the total intensity of the DAPI signal to correct the telomere signals for the amount of DNA that was in the evaluable tissue plane of the stained tissue sections. We confirmed that variability in DAPI signals, which, in theory would be higher in aneuploid cancer cells, did not explain our findings (data not shown).

The number of men who experienced progression to metastasis and prostate cancer death was relatively small especially when we divided the men into the four telomere biomarker groups. In the less variable/longer combination, only 1 man died of prostate cancer; this group had a reduced risk of the outcome and thus a deficit of events is expected. Indeed, if the four telomere biomarker groups each had had the same risk of poor outcome, then the number of prostate cancer deaths expected in each group each exceeded 5. While variability in telomere length among the cancer cells captured some of the same risk prediction as pathologic stage and grade, the telomere biomarker associations with outcome were independent of the currently used prognostic indicators and telomere biomarker added to the predictive capability of the prognostic indicators. The residual prediction suggests that the telomere biomarker may capture other features of disease aggressiveness that stage and grade do not capture.

In summary, we have identified that the combination of more variable telomere length among prostate cancer cells and shorter telomeres in prostate-cancer associated

stromal cells is potentially a new and independent tissue-based marker of prognosis in men surgically treated for clinically-localized prostate cancer, including in men with intermediate risk disease. Individually both telomere measurements are associated with an increased risk of lethal prostate cancer and prostate cancer death, but in combination (i.e. the telomere biomarker), these measurements are even more strongly positively associated with these outcomes. Future steps toward verifying the prognostic utility of the telomere biomarker include automating the assay for increased throughput and application to other cohorts of men. Also, future studies should address the utility of the telomere biomarker as a prognostic tool at the time of biopsy and in risk stratification for individualizing treatment and surveillance strategies.

MATERIALS AND METHODS

Study Population

The study population was drawn from men participating in the Health Professionals Follow-up Study (HPFS), an ongoing prospective cohort study on risk factors for cancer and other chronic diseases (https://www.hsph.harvard.edu/hpfs). In 1986, 51,529 men aged 40-75 years old enrolled. We asked them to complete a mailed questionnaire on their medical history and lifestyle factors at baseline and then again every two years. The conduct of the HPFS was approved by the Human Subjects Committee of the Harvard School of Public Health. The study on telomere length in prostate tissue and risk of aggressive prostate cancer was additionally approved by the Institutional Review Board at the Johns Hopkins Bloomberg School of Public Health.

Ascertainment of Prostate Cancer Cases and Their Follow-up

On each follow-up questionnaire, we asked the men to report a diagnosis of prostate cancer. We were able to obtain medical records and pathology reports pertaining to their diagnosis for 94.5% of the men who reported a prostate cancer diagnosis or for whom prostate cancer was mentioned on the death certificate. We abstracted TNM stage and PSA concentration at diagnosis from these records. We followed these men from the date of their diagnosis through January 2010 for the development of biochemical recurrence, distant metastasis, prostate cancer death, and non-prostate cancer death. The diagnosis of biochemical recurrence and distant metastasis (to bone or other organs) was collected by mailed questionnaire and then confirmed by the treating doctor. We learned of a participant's death from family members, the postal

system, or by searches of the National Death Index, which is estimated to have a sensitivity of more than 98% (25). Men were classified as having died from their prostate cancer if they had documented extensive metastatic disease. Follow-up for death is more than 98% complete for the HPFS cohort.

Confirmation of Pathologic Tumor Characteristics and Construction of Tissue Microarrays (TMAs)

After receiving participant permission, we requested tissue blocks of the prostatectomy specimens for the men who underwent surgical treatment for their prostate cancer from hospitals around the US. Study pathologists re-reviewed H&E-stained tissue sections containing prostate cancer and assigned a standardized Gleason sum as previously described (26); we used this Gleason sum in the analyses. We used five TMAs that were constructed for 631 prostate cancer cases as previously described (27). Briefly, a study pathologist selected the tumor focus that was the largest and/or had the highest Gleason sum, selected at least three areas of that focus, and then sampled them using 0.6 mm biopsy needles. For this analysis on telomere length, we excluded one man whose date of diagnosis and death were the same, men who had a prior history of a different primary cancer (N=7), and men who were diagnosed with prostate cancer incidentally after having undergone a transurethral resection of the prostate for the treatment of symptomatic benign prostatic hyperplasia (N=27). After these exclusions, 596 men were available for this analysis.

Measurement of Telomere Length Using FISH

FISH staining. Telomere length was assessed by telomere-specific fluorescence *in situ* hybridization (FISH) staining for telomeric DNA as previously described (9). Deparaffinized TMA slides were hydrated through a graded ethanol series, placed in deionized water, followed by deionized water plus 0.1% Tween-20. The TMA slides were steamed for 14 minutes (Black and Decker Handy Steamer Plus; Black and Decker) in citrate buffer (catalog No. H-3300; Vector Laboratories), removed and allowed to cool at room temperature for 5 minutes. The TMA slides were placed in PBS with Tween (PBST; catalog No. P-3563; Sigma) for 5 minutes, thoroughly rinsed with deionized water, followed by 95% ethanol for 5 minutes, and then air-dried. Twenty-five μL of a Cy3-labeled telomere-specific peptide nucleic acid hybridization probe (0.3 μg/mL peptide nucleic acid in 70% formamide, 10 mmol/L Tris, pH 7.5, 0.5% B/M Blocking reagent (catalog No. 1814-320; Boehringer-Mannheim) was applied, coverslipped, and denaturated by incubation for 4 minutes at 83°C. The TMA slides were then hybridized at room temperature for 2 hours in the dark. Following hybridization, the coverslips were then carefully removed and the slides were washed twice in peptide nucleic acid wash solution (70% formamide, 10 mmol/L Tris, pH 7.5, 0.1% albumin (from 30% albumin solution, catalog No. A-7284; Sigma) for 15 minutes each. The slides were rinsed in PBST followed by application of primary antibody (anti-cytokeratin antibody 34βE12, catalog no. 30904; Enzo Diagnostics, Farmingdale, NY) and incubated overnight at 4°C. After the incubation, the TMA slides were rinsed in PBST followed by application of fluorescent secondary antibody labeled with Alexa Fluor 488 (Molecular Probes) diluted 1:100 in Dulbecco's PBS, and incubated at room

temperature for 30 minutes. The TMA slides were then rinsed in PBST, thoroughly washed in deionized water, drained and counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (500 ng/mL in deionized water, Sigma Chemical Co. Cat #D-8417) for 5 minutes at room temperature. The TMA slides were then mounted with Prolong anti-fade mounting medium (catalog No. P-7481; Molecular Probes) and imaged. The peptide nucleic acid probe complementary to the mammalian telomere repeat sequence was obtained from Applied Biosystems. The probe has the sequence (N-terminus to C-terminus) CCCTAACCCTAACCCTAA with an N-terminal covalently linked Cy3 fluorescent dye. As a positive control for hybridization efficiency, a FITC-labeled peptide nucleic acid probe having the sequence ATTCGTTGGAAACGGGA with specificity for human centromeric DNA repeats (CENP-B binding sequence) was also included in the hybridization solution.

Microscopy. The TMA slides were imaged with a Nikon 50i epifluorescence microscope equipped with X-Cite series 120 illuminator (EXFO Photonics Solutions Inc., Ontario, CA) using a 40X/0.95 NA PlanApo lens with correction collar. Fluorescence excitation/emission filters are as follows: Cy3 excitation, 546 nm/10 nm BP; emission, 578 nm LP (Carl Zeiss Inc.); DAPI excitation, 330 nm; emission, 400 nm via an XF02 fluorescence set (Omega Optical, Brattleboro, VT); Alexa Fluor 488 excitation, 475 nm; emission, 535 via a B-2E/C filter set. For each color channel, separate grayscale images were captured using Nikon NIS-Elements software and an attached Photometrics CoolsnapEZ digital cooled CCD camera, and saved as 12-bit uncompressed TIFF files for use in downstream image analysis. Exposure times were

set such that fluorescence signal saturation was avoided. Integration times typically ranged from 400 to 800 milliseconds for Cy3 (telomere) and FITC (centromere) signal capture, 50 to 100 milliseconds for the DAPI nuclear counter-stain, and 100 ms to 400 ms for Alexa Fluor 488-conjugated antibodies. For cases with differing exposure times, arithmetic adjustment was made based on the known linear response characteristics of the imaging system. In all cases, telomeric signals were within the linear response range of the charge-coupled device camera, which was confirmed by use of fluorescent microbead intensity standards (InSpeck microscope image intensity calibration fluorescent microspheres; Molecular Probes).

Telomere length assessment. The digitized fluorescent telomere FISH signals were quantified using the open source, JAVA-based image analysis software package ImageJ (http://rsb.info.nih.gov/ij/) and a custom designed plugin ("Telometer"; http://demarzolab.pathology.jhmi.edu/telometer/). Matched telomeric and nuclear DNA grayscale TIFF image files were normalized by simple background subtraction, and the resulting telomere image was then run through a sharpening filter, followed by enhancement using a rolling ball algorithm for contouring of telomeric spots. A binarized mask of the telomere signals was then created and applied to the original unfiltered Cy3 telomere fluorescence image for data extraction. Data were recorded on an individual cell basis. For each cell, a region of interest was manually defined on the DAPI image by use of the freeform drawing tool in ImageJ. Guidance for cell type selection was provided by comparison to a separate 3-color merged image showing the combined DAPI, the telomere stain as well as the immunofluorescence stain; in this case

delineating benign prostatic basal cells. Telomeric signals identified by the binary segment mask, which were contained within the area inscribed by each circled nuclear DNA (DAPI) signal area, were then measured, and the data for each telomeric spot was tabulated. The total DAPI (DNA) fluorescence signal for each selected nucleus was likewise quantified. For each selected cell, the individual telomere intensities were summed ("telomere sum"), and this total was divided by the total DAPI fluorescence signal ("DAPI sum") for that same nucleus. This normalization to the nuclear DAPI signal corrects for differences in nuclear cutting planes and ploidy.

In TMA spots containing cancer, we evaluated prostate adenocarcinoma cells and the following cancer-associated cell types: benign-appearing prostate luminal epithelial, basal epithelial, and stromal (fibroblasts and smooth muscle). A small number of TMA spots did not contain cancer because of purposive sampling or because the cancer focus was exhausted during prior serial sectioning, leaving only benign-appearing tissue (N=133 men). In these TMA spots, we were able to evaluate benign-appearing prostate luminal epithelial, basal epithelial, and stromal cells. For each of the above cell types, we selected and analyzed 30 to 50 individual cells per man; not all cell types were available for evaluation for each man. For all TMA spots, other cell types, such as infiltrating lymphocytes, were excluded from the image analysis based on morphologic features. Tabulated data were stored in a MySQL (http://www.mysql.com) database and viewed through Microsoft Access (Microsoft Corp.).

Statistical Analysis

For each man and each of his cell types, we calculated (i) the median ratio of telomere

sum to DAPI sum as the measure of central tendency; (ii) the standard deviation and the 25^{th} to 75^{th}, 10^{th} to 90^{th}, and 5^{th} to 95^{th} percentile ranges as measures of variability from cell to cell; and (iii) the coefficient of variation (the standard deviation divided by the mean) as a standardized measure of variability. We divided the distribution of these measures into tertiles. We combined over adjacent tertiles that had similar associations. After viewing these results, we combined over telomere length in the CAS cells (shorter/longer) and variability in telomere length among the prostate cancer cells (more/less) to create four groups. We characterized the men by their demographic and prognostic indicators overall and by the combination of telomere length in CAS cells and variability in telomere length among the prostate cancer cells and tested for differences across the combinations using the chi-square test for proportions and one-way ANOVA for means. We determined whether length or variability in length differed across prognostic indicators – pathologic stage, prostatectomy Gleason sum, and PSA concentration at diagnosis.

To evaluate the association of telomere length, variability in length, and the telomere biomarker with prostate cancer outcomes and non-prostate cancer death, we generated two analytic cohorts. For progression to biochemical recurrence and lethal prostate cancer (defined as the subsequent development of distant metastasis or prostate cancer death), the analytic cohort consisted of men with clinically-localized disease without pathologic stage N1 or M1 (excluded N=7, total N=589 of which 560 were in the telomere biomarker analysis) at the time of prostatectomy. For prostate cancer death and non-prostate cancer death, the analytic cohort consisted of men with clinically-localized disease irrespective of pathologic stage (N=596). For each outcome,

we generated Kaplan-Meier curves for the four telomere biomarker groups and tested differences in the curves using the log-rank test. Separately by cell type, we estimated the hazard ratio (HR) and 95% confidence interval (CI) of each outcome using Cox proportional hazards regression. For telomere length in CAS cells and for telomere length variability among prostate cancer cells, we ran three models that were (i) adjusted for age (continuous) and calendar year (continuous) at diagnosis; (ii) further adjusted for prostatectomy Gleason sum (indicator variables: 3+4, 4+3, ≥8, versus ≤6 (reference)), pathologic TNM stage (≥T3b versus < T3b (reference)) and PSA at diagnosis (indicator variables: 10-20, >20, unknown, versus <10 ng/mL (reference)); and (iii) additionally mutually adjusted for telomere length and variability in length. For the combination of telomere length and variability in telomere length, we also ran models (i) and (ii). We calculated the C-statistic (28) for the model that included age and date of diagnosis, and the model the further included the prognostic indicators. Then, we added the telomere biomarker to these models. All analyses were performed using SAS v 9.2 (SAS Institute, Cary, NC). All statistical tests were two-sided, with $P<0.05$ considered to be statistically significant.

ACKNOWLEDGMENTS:

We thank Walter C. Willett, MD, DrPH, the Principal Investigator of the Health Professionals Follow-up Study, for has continued support. We would like to thank the participants and staff of the HPFS for their valuable contributions as well as the following state cancer registries for their help: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY. We also thank Dr. Michael C. Haffner for helpful discussions, and James Morgan in Dr. De Marzo's laboratory for his assistance with the telomere quantification software.

GRANT SUPPORT:

This study was supported by the Department of Defense grant W81XWH-05-1-0030 (Hopkins, Platz, PI) and additionally by Public Health Service research grant P50 CA58236 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services (Hopkins, William G. Nelson, PI; Pathology Core, De Marzo, Director). Dr. Heaphy was supported by Department of Defense grant W81XWH-09-1-0650 (Hopkins, Heaphy, PI). Dr. Joshu, the Martin D. Abeloff, MD Scholar-in-Training in Cancer Prevention, was supported by the Seraph Foundation. Dr. Kenfield was supported by the Prostate Cancer Foundation. The Health Professionals Follow-up Study was supported by Public Health Service research grants CA55075 and CA72036 (National Cancer Institute) and HL35464 (National Heart, Lung, and Blood Institute) from the National Institutes of Health, Department of Health and Human Services (Harvard, Willett, PI). Additional support for the study of prostate cancer in the HPFS includes CA133891 (Harvard, Giovannucci, PI) and CA141298 (Harvard, Stampfer, PI). The development of the HPFS prostate cancer tissue microarrays was supported by Department of Defense grant W81XWH-05-1-0562 (Harvard, Mucci, PI). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

REFERENCES

1. Blackburn EH. Telomeres. Trends Biochem Sci 1991;16:378-81.

2. Palm W, de Lange T. How shelterin protects mammalian telomeres. Annu Rev Genet 2008;42:301-34.

3. O'Sullivan RJ, Karlseder J. Telomeres: protecting chromosomes against genome instability. Nat Rev Mol Cell Biol 2010;11:171-81.

4. Meeker AK, Hicks JL, Platz EA, March GE, Bennett CJ, Delannoy MJ, *et al.* Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. Cancer Res 2002;62:6405-9.

5. Fordyce CA, Heaphy CM, Joste NE, Smith AY, Hunt WC, Griffith JK. Association between cancer-free survival and telomere DNA content in prostate tumors. J Urol 2005;173:610-4.

6. Treat EG, Heaphy CM, Massie LW, Bisoffi M, Smith AY, Davis MS, *et al.* Telomere DNA content in prostate biopsies predicts early rise in prostate-specific antigen after radical prostatectomy for prostate cancer. Urology 2010;75:724-9.

7. Donaldson L, Fordyce C, Gilliland F, Smith A, Feddersen R, Joste N, *et al.* Association between outcome and telomere DNA content in prostate cancer. J Urol 1999;162:1788-92.

8. Merlo LM, Maley CC. The role of genetic diversity in cancer. J Clin Invest 2010;120:401-3.

9. Meeker AK, Gage WR, Hicks JL, Simon I, Coffman JR, Platz EA, *et al.* Telomere length assessment in human archival tissues: combined telomere fluorescence in situ hybridization and immunostaining. Am J Pathol 2002;160:1259-68.

10. de Lange T. How telomeres solve the end-protection problem. Science 2009;326:948-52.

11. Vaziri H. Critical telomere shortening regulated by the ataxia-telangiectasia gene acts as a DNA damage signal leading to activation of p53 protein and limited life-span of human diploid fibroblasts. A review. Biochemistry (Mosc) 1997;62:1306-10.

12. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, *et al.* A DNA damage checkpoint response in telomere-initiated senescence. Nature 2003;426:194-8.

13. Maser RS, DePinho RA. Connecting chromosomes, crisis, and cancer. Science 2002;297:565-9.

14. Vukovic B, Park PC, Al-Maghrabi J, Beheshti B, Sweet J, Evans A, *et al.* Evidence of multifocality of telomere erosion in high-grade prostatic intraepithelial neoplasia (HPIN) and concurrent carcinoma. Oncogene 2003;22:1978-87.

15. Artandi SE, Chang S, Lee SL, Alson S, Gottlieb GJ, Chin L, *et al.* Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. Nature 2000;406:641-5.

16. Heaphy CM, Fleet TM, Treat EG, Lee SJ, Smith AY, Davis MS, *et al.* Organ-wide telomeric status in diseased and disease-free prostatic tissues. Prostate 2010;70:1471-9.

17. Joshua AM, Shen E, Yoshimoto M, Marrano P, Zielenska M, Evans AJ, *et al.* Topographical analysis of telomere length and correlation with genomic instability in whole mount prostatectomies. Prostate 2011;71:778-90.

18. Richardson AM, Woodson K, Wang Y, Rodriguez-Canales J, Erickson HS, Tangrea MA, *et al.* Global expression analysis of prostate cancer-associated stroma and epithelia. Diagn Mol Pathol 2007;16:189-97.

19. Ernst T, Hergenhahn M, Kenzelmann M, Cohen CD, Bonrouhi M, Weninger A, *et al.* Decrease and gain of gene expression are equally discriminatory markers for prostate carcinoma: a gene expression analysis on total and microdissected prostate tissue. Am J Pathol 2002;160:2169-80.

20. Ayala G, Tuxhorn JA, Wheeler TM, Frolov A, Scardino PT, Ohori M, *et al.* Reactive stroma as a predictor of biochemical-free recurrence in prostate cancer. Clin Cancer Res 2003;9:4792-801.

21. Cunha GR, Ricke W, Thomson A, Marker PC, Risbridger G, Hayward SW, *et al.* Hormonal, cellular, and molecular regulation of normal and neoplastic prostatic development. J Steroid Biochem Mol Biol 2004;92:221-36.

22. Sung SY, Chung LW. Prostate tumor-stroma interaction: molecular mechanisms and opportunities for therapeutic targeting. Differentiation 2002;70:506-21.

23. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol 2010;5:99-118.

24. Marusyk A, Almendro V, Polyak K. Intra-tumour heterogeneity: a looking glass for cancer? Nature Rev Cancer 2012;12:323-34.

25. Stampfer MJ, Willett WC, Speizer FE, Dysert DC, Lipnick R, Rosner B, *et al.* Test of the National Death Index. Am J Epidemiol 1984;119:837-9.

26. Stark JR, Perner S, Stampfer MJ, Sinnott JA, Finn S, Eisenstein AS, *et al.* Gleason score and lethal prostate cancer: does 3 + 4 = 4 + 3? J Clin Oncol 2009;27:3459-64.

27. Dhillon PK, Barry M, Stampfer MJ, Perner S, Fiorentino M, Fornari A, *et al.* Aberrant cytoplasmic expression of p63 and prostate cancer mortality. Cancer Epidemiol Biomarkers Prev 2009;18:595-600.

28. Kremers W. Concordance for Survival Time Data Including Time-Dependent Covariates Accounting for Ties in Predictor and Time, Technical Report 2006.

Table 1. Characteristics of prostate cancer cases at the time of prostatectomy, Health Professionals Follow-up Study

	All Men[†]	Telomere length variability among prostate cancer cells and telomere length in prostate cancer-associated stromal cells[‡]				
		Less variable/ Longer	More variable/ Longer	Less variable/ Shorter	More variable/ Shorter	*P*[1]
Number of men	596	98	91	280	98	
Mean ± standard deviation age at diagnosis (years)	65.3 ± 6.1	65.7 ± 6.4	65.6 ± 6.2	65.0 ± 6.0	65.9 ± 6.2	0.51
White (%)	91.1	87.8	89.0	93.9	87.8	0.12
Mean ± standard deviation year of diagnosis (years)	1994.2 ± 3.2	1994.7 ± 3.3	1994.6 ± 3.4	1993.8 ± 3.1	1994.5 ± 3.1	0.04
Prostatectomy Gleason sum (%)						
≤6	21.3	21.4	11.0	23.9	13.3	0.006
3+4	35.8	42.9	35.1	35.7	30.6	
4+3	24.8	23.5	29.7	25.4	26.5	
≥8	18.1	12.2	24.2	15.0	29.6	
Pathologic stage ≥T3b (%)	12.6	5.1	18.7	12.1	17.3	0.02
Serum PSA concentration at diagnosis in ng/mL (%)						
<10	59.2	73.5	54.9	57.5	54.1	0.17
10-20	17.6	14.3	17.6	17.5	20.4	
>20	10.1	4.1	14.3	10.7	11.2	
Unknown	13.1	8.1	13.2	14.3	14.3	

[1] From a chi-square test for proportions and a one-way ANOVA for means
[†] Among all men
[‡] Among men for whom telomere length could be determined for both prostate cancer cells and prostate cancer-associated stromal cells (N=567)

Table 2. Association of more variable telomere length among prostate cancer cells and shorter telomere length in prostate cancer-associated stromal cells with risk of biochemical recurrence, lethal prostate cancer, prostate cancer death, and non-prostate cancer death, Health Professionals Follow-up Study*

	Biochemical recurrence†		Lethal prostate cancer†		Prostate cancer death		Non-prostate cancer death	
	More variable length among cancer cells	Shorter length in associated stromal cells	More variable length among cancer cells	Shorter length in associated stromal cells	More variable length among cancer cells	Shorter length in associated stromal cells	More variable length among cancer cells	Shorter length in associated stromal cells
Age and year of diagnosis adjusted								
HR	1.88	0.92	2.56	1.95	3.07	2.37	1.29	0.73
95% CI	(1.39-2.55)	(0.67-1.26)	(1.50-4.37)	(1.01-3.78)	(1.71-5.51)	(1.11-5.08)	(0.91-1.82)	(0.52-1.02)
Multivariable adjusted‡								
HR	1.51	0.96	1.64	2.43	1.78	2.94	1.25	0.73
95% CI	(1.11-2.07)	(0.70-1.31)	(0.94-2.86)	(1.24-4.76)	(0.96-3.30)	(1.35-6.39)	(0.88-1.78)	(0.52-1.02)
Additionally mutually adjusted§								
HR	1.55	1.10	2.21	3.39	2.39	4.18	1.17	0.76
95% CI	(1.12-2.15)	(0.79-1.54)	(1.24-3.95)	(1.65-6.98)	(1.26-4.51)	(1.8-9.67)	(0.81-1.68)	(0.54-1.08)

* Associations are reported as hazard ratios (HR) and 95% confidence intervals (95% CI) for the following comparisons: more variable (top tertile of variability) in telomere length among prostate cancer cells versus less variable (bottom and middle tertiles), and shorter (shortest and middle tertiles) median telomere length in CAS cells versus longer (longest tertile).

† Restricted to men without metastatic prostate cancer at the time of diagnosis.

‡ Adjusted for age (continuous) and year (continuous) of diagnosis, prostatectomy Gleason sum (categorical: ≤6, 3+4, 4+3, ≥8), pathologic TNM stage (categorical ≥T3b) and serum PSA concentration at diagnosis (categorical: <10, 10-20, >20 ng/mL, unknown).

§ Association with more variable telomere length among prostate cancer cells further adjusted for shorter telomere length in prostate cancer-associated stromal cells; association with shorter telomere length in prostate cancer-associated stromal cells further adjusted for more variable telomere length among prostate cancer cells.

Table 3. Association of the telomere biomarker* with risk of biochemical recurrence, lethal prostate cancer, prostate cancer death, and non-prostate cancer death, Health Professionals Follow-up Study

Outcome	Events/ Person-years	Age and year of diagnosis adjusted			Multivariable adjusted‡		
		HR	(95% CI)	*P*	HR	(95% CI)	*P*
Biochemical recurrence†							
Less variable/Longer	22/1,083	1.00	(reference)	--	1.00	(reference)	--
More variable/Longer	35/841	1.96	(1.15-3.34)	0.014	1.42	(0.82-2.45)	0.21
Less variable/Shorter	74/3,149	1.12	(0.69-1.80)	0.66	1.02	(0.63-1.66)	0.93
More variable/Shorter	40/880	2.12	(1.26-3.57)	0.005	1.67	(0.98-2.83)	0.06
Lethal prostate cancer†,§							
Less variable/Longer	2/1,304	1.00	(reference)	--	1.00	(reference)	--
More variable/Longer	8/1,137	4.49	(0.95-21.16)	0.06	2.48	(0.52-11.93)	0.26
Less variable/Shorter	24/3,733	3.93	(0.93-16.66)	0.06	3.74	(0.88-15.96)	0.07
More variable/Shorter	20/1,148	12.31	(2.87-52.75)	0.0007	8.12	(1.88-34.97)	0.005
Prostate cancer death§							
Less variable/Longer	1/1,312	1.00	(reference)	--	1.00	(reference)	--
More variable/Longer	6/1,163	6.68	(0.80-55.55)	0.08	3.76	(0.44-31.79)	0.22
Less variable/Shorter	19/3,822	6.21	(0.83-46.46)	0.08	6.23	(0.82-47.06)	0.08
More variable/Shorter	20/1,194	24.59	(3.29-183.62)	0.002	14.10	(1.87-106.49)	0.01
Non-prostate cancer death							
Less variable/Longer	28/1,312	1.00	(reference)	--	1.00	(reference)	--
More variable/Longer	30/1,163	1.29	(0.77-2.17)	0.33	1.21	(0.71-2.07)	0.48
Less variable/Shorter	65/3,822	0.81	(0.52-1.26)	0.35	0.78	(0.50-1.23)	0.28
More variable/Shorter	21/1,194	0.92	(0.52-1.62)	0.76	0.88	(0.49-1.57)	0.66

* The combination of variability in telomere length among prostate cancer cells and telomere length in prostate cancer-associated stromal cells.
† Restricted to men without metastatic prostate cancer at the time of diagnosis.
‡ Adjusted for age (continuous) and year (continuous) of diagnosis, prostatectomy Gleason sum (categorical: ≤6, 3+4, 4+3, ≥8), pathologic TNM stage (categorical ≥T3b) and serum PSA concentration at diagnosis (categorical: <10, 10-20, >20 ng/mL, unknown).
§ Using the group with the largest sample size – less variable/shorter – as the reference, the HRs are as follows: lethal prostate cancer – less variable/longer 0.27 (*P*=0.07), less variable/shorter 0.66 (*P*=0.33), more variable/shorter 2.17 (*P*=0.02); death from prostate cancer - less variable/longer 0.16 (*P*=0.08), less variable/shorter 0.61 (*P*=0.30), more variable/shorter 2.26 (*P*=0.02).

Table 4. Hazard ratios (HR) of lethal prostate cancer and prostate cancer death for the telomere biomarker*and the currently used prognostic characteristics, Health Professionals Follow-up Study

Outcome	HR‡ (95% CI)	*P*
Lethal Prostate Cancer†		
Telomere biomarker		
Less variable/Longer	1.00 (ref)	--
More variable/Longer	2.48 (0.52-11.93)	0.26
Less variable/Shorter	3.74 (0.88-15.96)	0.07
More variable/Shorter	8.12 (1.88-34.97)	0.005
Prostatectomy Gleason sum		
≤6	0.27 (0.03-2.17)	0.22
3+4	1.00 (ref)	--
4+3	3.77 (1.59-8.96)	0.003
≥8	3.86 (1.57-9.49)	0.003
Pathologic stage ≥T3b	4.20 (2.27-7.75)	<0.0001
Serum PSA concentration at diagnosis (ng/mL)		
<10	1.00 (ref)	--
10-20	1.13 (0.52-2.45)	0.76
>20	1.12 (0.48-2.58)	0.80
Unknown	1.55 (0.68-3.55)	0.30
Prostate Cancer Death		
Telomere biomarker		
Less variable/Longer	1.00 (ref)	--
More variable/Longer	3.76 (0.44-31.79)	0.22
Less variable/Shorter	6.23 (0.82-47.06)	0.08
More variable/Shorter	14.10 (1.87-106.49)	0.01
Prostatectomy Gleason sum		
≤6 §	0.00 --	0.99
3+4	1.00 (ref)	--
4+3	2.93 (1.04-8.25)	0.04
≥8	4.45 (1.6-12.44)	0.004
Pathologic stage ≥T3b	5.08 (2.63-9.84)	<0.0001
Serum PSA concentration at diagnosis (ng/mL)		
<10	1.00 (ref)	--
10-20	0.71 (0.26-1.94)	0.50
>20	1.19 (0.47-3)	0.71
Unknown	2.83 (1.21-6.63)	0.02

* The combination of variability in telomere length among cancer cells and telomere length in cancer-associated stromal cells.
† Restricted to men without metastatic prostate cancer at the time of diagnosis.
‡ Mutually adjusted and adjusted for age (continuous) and year (continuous) of diagnosis.
§ No prostate cancer deaths occurred in these men. Combining ≤6 and 3+4 as the reference, the HRs of prostate cancer death were 4.54 (95% CI 1.60-12.91; *P*=0.004) for 4+3, and 6.84 (95% CI 2.42-19.34; *P*=0.0003) for ≥8. The HRs of prostate cancer death were unchanged for the telomere biomarker (Less variable/Longer: HR=1.00 (ref); More variable/Longer: HR=3.80 (95% CI 0.45-32.13; *P*=0.22); Less variable/Shorter: HR=6.19 (95% CI 0.82-46.74; *P*=0.08); More variable/Shorter: HR=14.21 (95% CI 1.88-107.28; *P*=0.01)).

FIGURE LEGENDS

Figure 1. Telomere-specific FISH in prostate adenocarcinomas. Panels A–D show examples of telomere length and cell-to-cell variability in telomere length in malignant and benign prostate tissue from men in the Health Professionals Follow-up Study who were surgically treated for clinically-localized prostate cancer. **(A)** This case has strikingly variable telomere signals among the cancer cells. **(B)** This case has extremely short telomere signals and low variability in telomere length from cancer cell to cancer cell. **(C)** This case has weak telomere signals in the cancer-associated stromal cells. **(D)** This case has strong telomere signals in cancer-associated stromal cells. In all the images, the DNA is stained with DAPI (blue) and telomere DNA is stained with the Cy3-labeled telomere-specific peptide nucleic acid probe (red). Of note, the centromere DNA, stained with the FITC-labeled centromere-specific peptide nucleic acid probe, has been omitted from the image to emphasize the differences in the telomere lengths. In all panels, the asterisks highlight the cancer cells and the arrows point to the cancer-associated stromal cells. Original magnification × 400.

Figure 2. Prostate cancer outcome-specific survival and non-prostate cancer survival by the telomere biomarker combination of more variability in telomere length among prostate cancer cells and shorter telomeres in prostate cancer-associated stromal cells, Health Professionals Follow-up Study. (A) With respect to biochemical recurrence, men with the more variable/shorter and more variable/longer combinations had similarly higher risk over time, men with the less variable/longer

combination had the lowest risk, and men with the less variable/shorter combination had an intermediate risk. **(B and C)** With respect to lethal prostate cancer and death from prostate cancer, men with the more variable/shorter combination were the most likely to experience these outcomes over time, whereas the men with the less variable/longer combination were the least likely to experience these outcomes, and men with the other two combinations had intermediate risk. **(D)** With respect to non-prostate cancer death, the telomere biomarker was not associated with risk, supporting the specificity of the biomarker for prostate cancer outcomes.

Figure 1. Telomere-specific FISH in prostate adenocarcinomas.

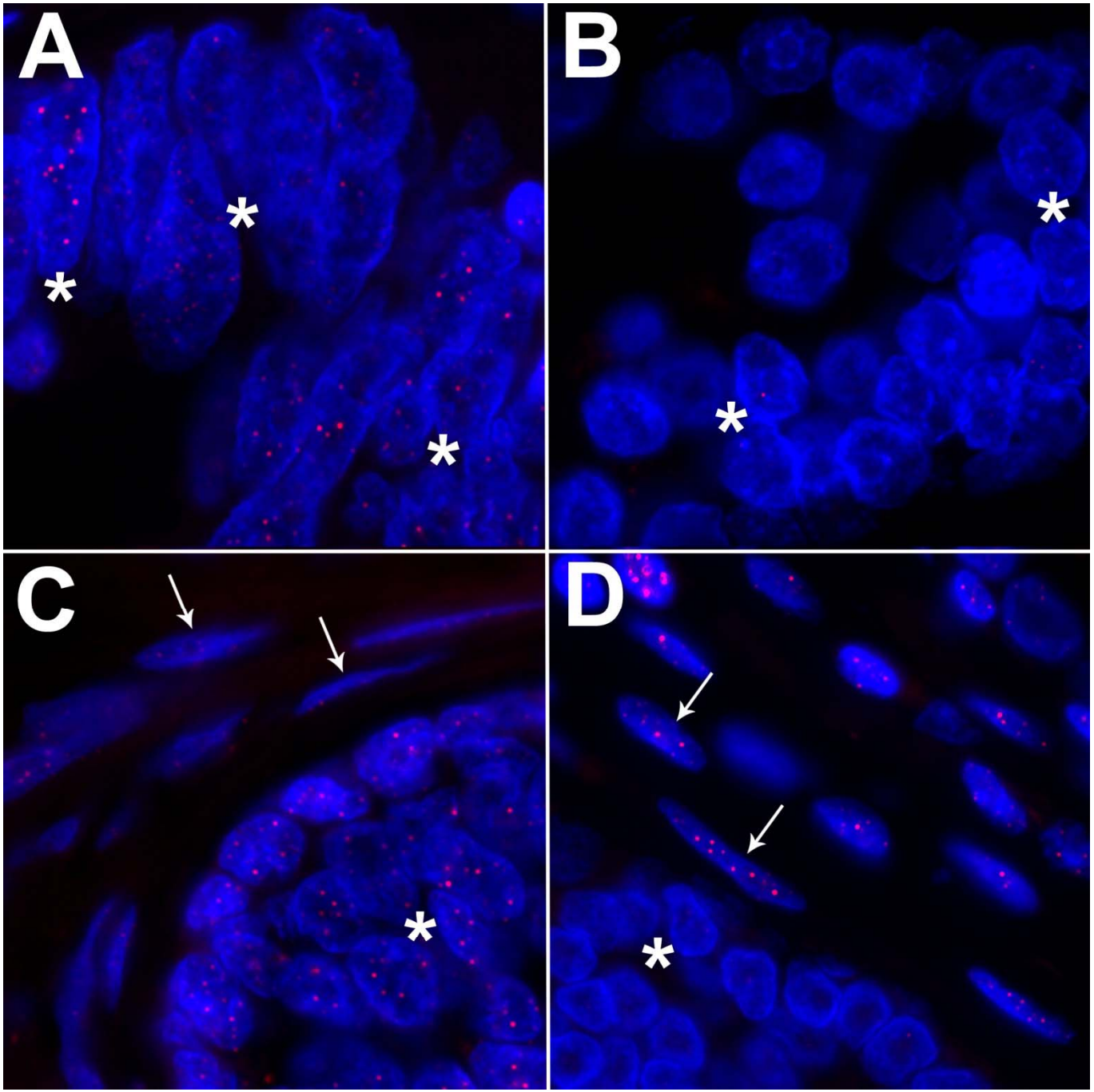
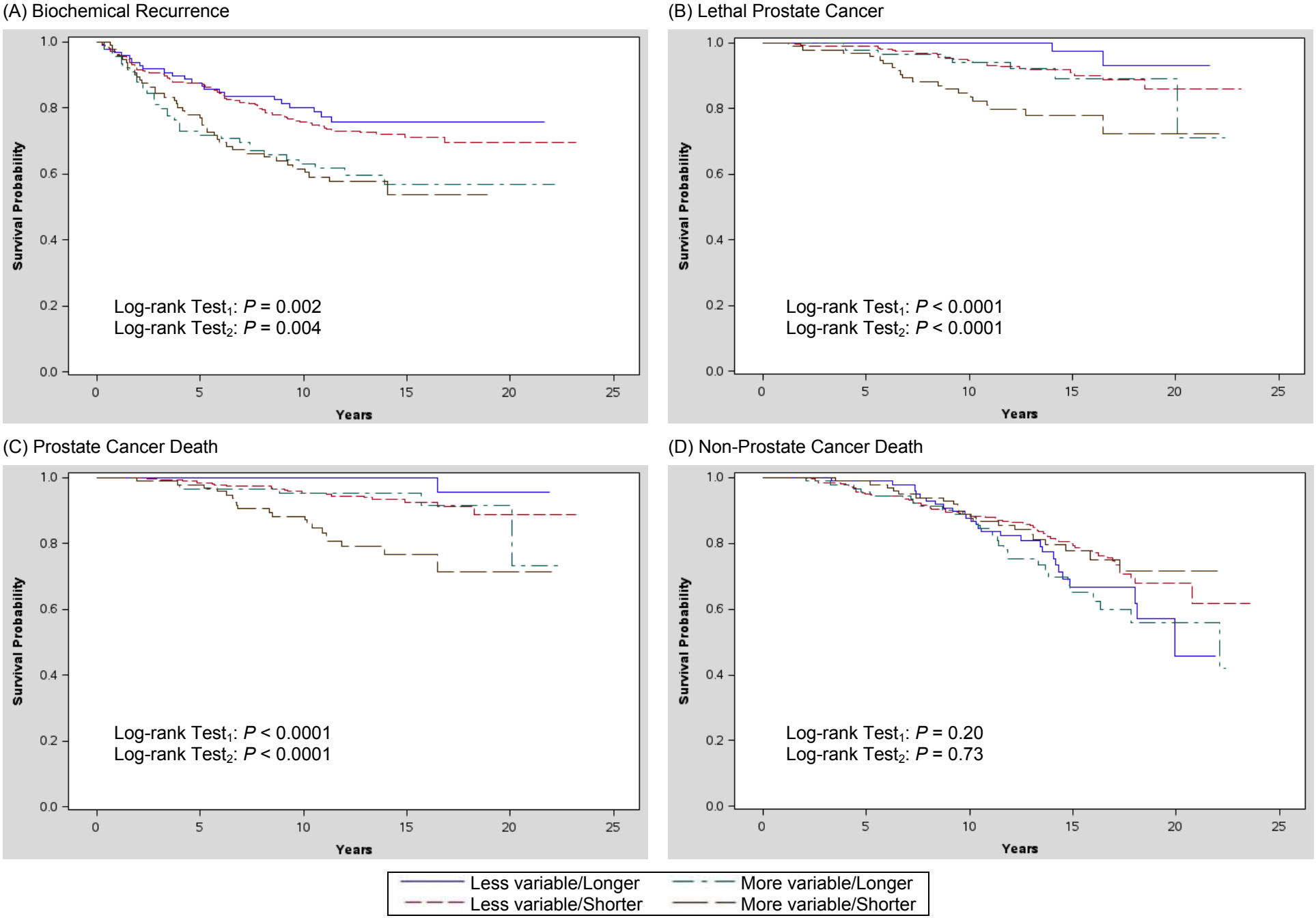


Figure 2. Prostate cancer outcome-specific survival and non-prostate cancer survival by the telomere biomarker combination of more variability in telomere length among prostate cancer cells and shorter telomeres in prostate cancer-associated stromal cells, Health Professionals Follow-up Study



Log-rank Test$_1$: compares the survival distributions across all 4 telomere biomarker categories
Log-rank Test$_2$: compares the survival distributions of men with the less variable/longer combination to men with more variable/shorter combination of the telomere biomarker